Stomatal density profiling in *Vitis vinifera* L. using non-destructive field microscopy

by

TL Venter

Thesis presented in partial fulfilment of the requirements for the degree of
Master of Agricultural Science

at

Stellenbosch University

Department of Viticulture and Oenology, Faculty of AgriSciences

Supervisor: Dr AE Strever

December 2015

Stellenbosch University https://scholar.sun.ac.za

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2015

Copyright © 2015 Stellenbosch University
All rights reserved

Stellenbosch University https://scholar.sun.ac.za

SUMMARY

When plants began colonising land ca. 400 million years ago, they needed to adapt structurally to limit water loss from aerial parts. Thus, a waxy cuticle developed on these parts, particularly the leaves, in order to prevent transpiration from these surfaces. This layer is, however, impermeable to carbon dioxide (CO_2) which is required as a substrate in photosynthesis. Therefore, the stomata evolved to allow for gaseous exchange to take place. The main function of stomata is to ensure that the amount of CO_2 taken up by the plant is balanced with the amount of water available to it. Stomatal development and function has been studied extensively, but few of these studies have been done on *Vitis vinifera* L. Since the stomatal development process is complex and carefully guided, a lot of these past studies were conducted on the model plant *Arabidopsis thaliana* in order to attempt to ascertain which genes may be involved in this process, and how. Environmental stimuli have been found to affect both stomatal development and function. These effects can be short-term, in which case stomata will respond to a momentary change in conditions by opening or closing the stomatal pore (change in stomatal function), or long-term, by which the response is more permanent and affects stomatal density and/or size (change in stomatal development). Such factors which have been investigated include CO_2 levels, relative humidity, both light quantity and quality, as well as limited water availability. It has been found that changes in response to water-stress are brought about by the increased production of the plant hormone, abscisic acid, in the roots, which is then transported to the leaves in the transpiration stream. For *Vitis vinifera* L. the effect of light (quantity and quality), CO_2 concentration and water-stress on stomatal development and function have been investigated by other researchers.

Various methods are used in stomatal research. The most common methods are light and scanning electron microscopy. These methods are both destructive and make use of intact leaf tissue, or epidermal peels and impressions. In this study an adapted microscopy technique was used in order to test whether it would be suitable for conducting stomatal investigations non-destructively over a period of time. Four *Vitis vinifera* L. cultivars were selected for this study and in-field stomatal investigations were carried out over the period between bunch closure and post-véraison. A portable digital microscope was used to capture images and these were then digitally analysed. The aim was to investigate whether stomatal density differs between cultivars, leaves of a single plant as well as between different positions on a single leaf. In general there were differences found between cultivars, but not all the differences were significant. Younger leaves displayed a higher stomatal density than more mature leaves and the degree of this also varied between cultivars. Little differences were noted over time and between on-leaf positions.

The method was successful in conducting the relevant investigations, but it was not without problems and shortcomings. The resolution of the images produced was not sufficient to allow for the calculation of stomatal index and size, but stomatal density could be determined reliably. With the rate at which new technology becomes available, these issues may be minimised or eliminated in the near future, and the application of this method to stomatal investigations expanded.

Stellenbosch University https://scholar.sun.ac.za

OPSOMMING

Sedert plante sowat 400 miljoen jaar gelede die aarde se oppervlak begin bedek het, moes hulle struktureel aanpas om die waterverlies vanaf bo-grondse dele te verminder. Sodoende het die waslaag wat op sulke plantdele, veral die blare, voorkom, ontstaan. Hierdie laag is egter ook nie deurlaatbaar vir koolsuurgas (CO_2) nie, wat benodig word vir fotosintese. Huidmondjies het dus ontwikkel om die gas-uitruilingsproses moontlik te maak. Die hoofdoel van die huidmondjies is om die hoeveelheid CO_2 wat deur die plant opgeneem word, met die hoeveelheid water beskikbaar tot die plant, te balanseer. Daar is reeds baie navorsing gedoen oor huidmondjie-ontwikkeling en -funksie, maar min studies is spesifiek op *Vitis vinifera* L. gedoen. Aangesien die ontwikkelingsproses van huidmondjies baie kompleks is en noukeurig gereguleer word, is vele studies op die modelplant, *Arabidopsis thaliana*, uitgevoer in ‘n poging om te probeer uitvind watter gene moontlik die proses beheer, sowel as die manier waarop hierdie regulering bewerkstellig word. Daar is bevind dat beide ontwikkeling en funksie van huidmondjies deur omgewingsfaktore beïnvloed word. Hierdie veranderings kan óf oor die korttermyn geskied deur die opening, of sluiting van die huidmondjies (‘n aanpassing in huidmondjiefunksie), óf op ‘n langtermyn basis deur ‘n verandering in huidmondjiedigtheid en/of -grootte (‘n aanpassing met betrekking tot huidmondjie-ontwikkeling). Laasgenoemde is ‘n meer permanente aanpassing. Sulke omgewingsfaktore wat al in studies gemonitor is sluit in CO_2-vlakke, relatiewe humiditeit, ligkwantiteit en -kwaliteit, sowel as watertekort-toestande. Daar is gevind dat laasgenoemde ‘n verandering in huidmondjies teweegbring deur middel van die verhoogde produksie van absisiensuur in die wortels. Hierdie hormoon word dan in die transpirasiestroom na die blare toe vervoer waar die effek bewerkstellig word. Die effek van ligkwaliteit en -kwantiteit, sowel as CO_2-vakke en watertekort-toestande op huidmondjie-ontwikkeling en -funksie is al vir *Vitis vinifera* L. nagevors.

Verskeie metodes word in die navorsing van huidmondjies gebruik, waarvan ligmikroskopie en skandeer-elektronmikroskopie die mees algemeen is. Beide hierdie metodes is destruktief en maak gebruik van blaarweefsel, epidermale afdrukke of afgeskilde lagies. In hierdie studie is ‘n aangepaste mikroskopiese metode gebruik om vas te stel of dit suksesvol toegepas kan word om nie-destruktiewe waarnemings van huidmondjies oor ‘n tydperk te kan uitvoer. Vier *Vitis vinifera* L. kultivars is vir die studie gebruik en metings is oor die tydperk vanaf trossluiting tot na deurslaan gedoen. ‘n Draagbare digitale mikroskoop is gebruik om beelde te neem wat later digitaal geanaliseer kon word. Die doel was om vas te stel of huidmondjiedigtheid tussen kultivars verskil, so wel as om te bepaal of daar variasies hiervan tussen verkillende blare op ‘n enkele plant en ook oor posisies op ‘n enkele blaar is. In die algemeen het kultivars van mekaar verskil, maar die verskille was nie almal beduidend nie. Jonger blare het ‘n hoër huidmondjiedigtheid getoon as die meer volwasse blare. Daar was nie veel variasie in huidmondjiedigtheid oor tyd, of tussen die verskillende posisies op die blare nie.

Die metode kon suksesvol toegepas word om die beoogde waarnemings te maak, maar daar was tog probleme en tekortkominge. Die resolusie van die beelde wat verkry is was nie hoog genoeg om die bepaling van huidmondjie-grootte en -indeks moontlik te maak nie, maar huidmondjiedigtheid kon effektief bepaal word. Gegewe die tempo waarteen nuwe tegnologie ontwikkel, kan dit moontlik wees om hierdie probleem in die nabye toekoms aan te spreek. Die toepassing van hierdie metode vir die navorsing van huidmondjies mag dan sodoende uitgebrei word.

Stellenbosch University https://scholar.sun.ac.za

This thesis is dedicated to my family for their love, support and encouragement

Stellenbosch University https://scholar.sun.ac.za

BIOGRAPHICAL SKETCH

Talitha Laetitia Venter was born in Johannesburg on 23 March 1987. She matriculated at Collegiate Girls' High School, Port Elizabeth in 2005. Talitha enrolled at Stellenbosch University in 2006 and obtained the degree BScAgric in Viticulture and Oenology in December 2009. In January 2010, she became viticulturist at Thelema Mountain Vineyards in Stellenbosch and managed the vineyards on both the Stellenbosch and Elgin properties. In 2013, Talitha decided to enrol for her MScAgric in Viticulture at Stellenbosch University on a part-time basis. In June of 2013 she took up a position at the Department of Viticulture and Oenology, Stellenbosch University, as a Technical Officer: Viticulture. She is currently employed as Technical Officer: Viticulture for the Institute of Grape and Wine Sciences, Department of Viticulture and Oenology, Stellenbosch University.

Stellenbosch University https://scholar.sun.ac.za

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- **Dr AE Strever,** Department of Viticulture and Oenology, Stellenbosch University, for his guidance, encouragement and motivation during my study.

- **Prof MA Vivier,** Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, for her valuable inputs, advice and crucial inspiration.

- **Prof M Kidd,** Centre for Statistical Consultation, Stellenbosch University, for his assistance with the statistical analysis of the data.

- **Mr Gerard Martin,** Institute for Grape and Wine Sciences, Department of Viticulture and Oenology, Stellenbosch University, for allowing me study leave in order to finish this thesis.

- **Miss Larissa van der Vyver,** for her invaluable assistance in the field, friendship and encouragement.

- **My family Mr Ben Venter, Mrs Laetitia Venter and Mr Gustav Venter** for their continued support in my endeavours and for encouraging me throughout this process. A special word of thanks to my father for his assistance in the field, and to my mother for the translation of German literature, and the proof reading and editing of this thesis. To my brother, thank you for being my best friend and being there every step of the way.

- **My godparents Mr Tjaart Coetzee and Mrs Martinette Coetzee,** for their interest in my progress, and their love and support.

- **My friends and colleagues** at the Department of Viticulture and Oenology, Stellenbosch University, for their kind words of encouragement, advice and contributions.

Stellenbosch University https://scholar.sun.ac.za

PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the South African Journal of Enology and Viticulture.

Chapter I **General introduction and project aims**

Chapter II **Literature review**
Stomatal development and function: current knowledge and research methods with reference to *Vitis vinifera* L. and other plant species

Chapter III **Methodology**
Experiment layout and the use of field microscopy to investigate stomatal density non-destructively

Chapter IV **Research results**
Stomatal density and stomatal number per leaf investigated in four cultivars of *Vitis vinifera* L.

Chapter V **General discussion and conclusions**

Stellenbosch University https://scholar.sun.ac.za

CONTENTS

Stellenbosch University https://scholar.sun.ac.za

Stellenbosch University https://scholar.sun.ac.za

Chapter 1

Introduction and project aims

Stellenbosch University https://scholar.sun.ac.za

CHAPTER I: INTRODUCTION AND PROJECT AIMS

1.1 Introduction

Stomata are key to the survival of all plants, not only because they allow for gaseous exchange to take place, but also because they are central to a plant's adaptation to changing or unfavourable conditions. The aim is always for photosynthesis to be balanced with the amount of water that is available to the plant (Payne, 1979; Chaerle *et al.*, 2005; Casson & Gray, 2008). Short-term responses to unfavourable conditions will be for the plant to alter the stomatal aperture, but when such conditions prevail, a change in stomatal density or size may occur (Casson & Gray, 2008).

By investigating factors that affect stomatal development, some insight can be gained as to how plants will adapt to certain conditions. Many studies have been conducted to investigate the effect of environmental factors, such as CO_2 levels, humidity and light intensity (as well as quality), on stomatal density and size. In an attempt to unravel the mechanisms behind these adaptations, such studies are often conducted on a molecular level and under controlled conditions using the model plant *Arabidopsis thaliana* in particular (Pillitteri & Torii, 2012), but it is an intricate and complex series of responses. Few studies have been carried out on *Vitis vinifera*. Rogiers *et al*. (2011) investigated the effect of soil temperature and atmospheric CO_2 on stomatal density using potted Chardonnay vines – this study was the first of its kind. In another study the effect of light intensity on stomatal density of primary and lateral leaves was investigated for field grown Cabernet franc and Trebbiano Toscano vines (Palliotti *et al.*, 2000). In a study by Düring (1980) the stomatal density of different *Vitis* species and cultivars were investigated.

The effect of biotic factors, such as plant vigour, leaf size, leaf age and hormones will also play a role in regulating stomatal functioning and development. It is, however, difficult to separate the biotic and abiotic influences completely, since changes in for example growth habit of the plant will inevitably lead to changes in light conditions and other microclimatic parameters.

Most studies investigating stomatal development by the counting and measuring of stomata, employ scanning electron microscopy (SEM) on leaf sections, or general microscopy on leaf imprints. These are both destructive methods. By establishing other non-destructive methods it opens the door to long-term (seasonal) monitoring of stomatal development on the same leaves or even real-time monitoring of stomata. This study aimed to determine whether field microscopy could be used for such repeated measurements.

In addition to method description, this study further aimed to establish whether biotic factors, such as cultivar and leaf position, affect stomatal density and if so, to what degree. If this study proves field microscopy to be a viable means of conducting stomatal investigations, the possibilities for its application would be close to limitless. By investigating stomata while taking field conditions and cultivation practices into account, along with environmental measurements (light, water status and temperature), the process of stomatal development and how it is regulated may become more apparent.

The project was complex and posed many challenges as the unfamiliarity and technical issues with the hardware and software used had to be overcome. Boundaries had to be set on how many factors could be investigated in order to reach realistic goals.

Stellenbosch University https://scholar.sun.ac.za

1.2 Project Aims

The aims of this study were:

Aim 1: To describe a new non-destructive method for stomatal investigations - *assessing the use of an adapted microscopy method for analysing stomatal density in Vitis vinifera* in the field

Objective 1 – determining whether field microscopy using a digital microscope is a viable tool for investigating stomatal density non-destructively.

Objective 2 – determining the most effective way of analysing the images obtained from field microscopy using image editing software.

Aim 2: To gain more knowledge about the variation of stomatal density and stomatal number per leaf in *Vitis vinifera* with regards to:

- *Time of season*
- *Cultivar*
- *Leaf position (on the shoot)*
- *On-leaf (intra-leaf) observation position (for stomatal density only)*
- *Leaf size (for stomatal number per leaf only)*

1.3 Literature cited

Casson, S.A. & Gray, J.E., 2008. Influence of environmental factors on stomatal development. New Phytol. 178(1), 9–23.

Chaerle, L., Saibo, N. & Van Der Straeten, D., 2005. Tuning the pores: towards engineering plants for improved water use efficiency. Trends Biotechnol. 23(6), 308–315.

Düring, H., 1980. Stomatafrequenz bei Blättern von *Vitis*-Arten und -Sorten. Vitis 19, 91–98.

Palliotti, A., Cartechini, A. & Ferranti, F., 2000. Morpho-anatomical and physiological characteristics of primary and lateral shoot leaves of Cabernet Franc and Trebbiano Toscano grapevines under two irradiance regimes. Am. J. Enol. Vitic. 51(2), 122–130.

Payne, W.W., 1979. Stomatal patterns in embryophytes: their evolution, ontogeny and interpretation. Taxon 28(1), 117–132.

Pillitteri, L.J. & Torii, K.U., 2012. Mechanisms of stomatal development. Annu. Rev. Plant Biol. 63, 591–614.

Rogiers, S.Y., Hardie, W.J. & Smith, J.P., 2011. Stomatal density of grapevine leaves (*Vitis vinifera* L.) responds to soil temperature and atmospheric carbon dioxide. Aust. J. Grape Wine Res. 17(2), 147–152.

Stellenbosch University https://scholar.sun.ac.za

Chapter 2

Literature review

Stomatal development and function: current knowledge and research methods with reference to *Vitis vinifera* L. and other plant species

Stellenbosch University https://scholar.sun.ac.za

CHAPTER II: STOMATAL DEVELOPMENT AND FUNCTION: CURRENT KNOWLEDGE AND RESEARCH METHODS WITH REFERENCE TO *VITIS VINIFERA* L. AND OTHER PLANT SPECIES

2.1 Introduction

Stomata are the microscopic pores found in the epidermis of almost all land plants. When plants began colonising land some 400 million years ago (Martin *et al.*, 1983), they had to be protected from desiccation. Plants also needed to be able to survive across a range of fluctuating environmental conditions on land (Hetherington & Woodward, 2003). This led to the development of a waxy cuticle on plant aerial parts. While creating a barrier to water loss, this cuticle also prevented the uptake of carbon dioxide (CO_2) which is necessary for photosynthesis. Thus stomata evolved in the epidermis to serve as a passage through which gaseous exchange between the plant intercellular spaces and the environment could take place (Martin *et al.*, 1983; Hetherington & Woodward, 2003). They occur mostly in, but are not limited to, the epidermis of the leaves and can be found on flowers, fruits, green stems and tendrils as well (Martin *et al.*, 1983). Although stomatal pores comprise not more than 5% of the total leaf surface when fully open (Martin *et al.*, 1983; Weyers & Meidner, 1990; Hetherington & Woodward, 2003), they could account for water loss from the leaf as high as 95% (Kramer & Boyer, 1995). A very small amount of gaseous exchange is able to take place across the epidermis when the stomata are completely closed (Boyer *et al.*, 1997), but the water loss through this path may be as high as 70% if there was no cuticle present (Hetherington & Woodward, 2003).

It is important for gaseous exchange to be optimal, with the amount of carbon assimilated being balanced with the amount of water that is available to a plant. This is the main function of stomata (Raven, 2002) and they will adapt under unfavourable climatic conditions, such as water-stress, to ensure this. The ability of plants to adapt to their environment is of utmost importance to their survival for the mere fact that they are sessile and therefore unable to move to more favourable conditions.

Stomata have been the subject of numerous studies to investigate their developmental changes in response to environmental stimuli. Many of the studies have been conducted on a molecular level, especially in the model plant *Arabidopsis thaliana* (Nadeau & Sack, 2002; Pillitteri & Torii, 2012), in order to ascertain mechanisms involved in guiding stomatal development and responses. Other studies are more concerned with the physical characteristics of stomata, such as stomatal size and stomatal density.

Stomatal studies in *Vitis vinifera* have been limited, however. The effect of light intensity on stomatal density has been investigated for both primary and lateral shoot leaves (Palliotti *et al.*, 2000). The effect of atmospheric CO_2 and soil water status was determined by Rogiers *et al.* (2011) and this investigation was the first of its kind in grapevines. Differences in stomatal density have been noticed between cultivars grown under similar conditions (Palliotti *et al.*, 2000) and this would be a good field of study to identify cultivar suitability to certain environmental conditions as well as cultivar adaptability to a changing climate.

Stellenbosch University https://scholar.sun.ac.za

2.2 Stomatal development

2.2.1 Basics of stomatal structure

Stomata consist of two specialised epidermal cells, the guard cells, which surround the stomatal opening (pore). The guard cells and surrounding epidermal cells are often referred to as the stomatal complex. A diagram of a stoma is shown in Figure 1. The associated epidermal cells are termed neighbouring cells if they are identical to other epidermal cells or subsidiary cells if they can be distinguished from these other cells. Below the guard cells and stomatal pore is a gap in the mesophyll tissue – the sub-stomatal chamber (Martin *et al.*, 1983; Weyers & Meidner, 1990).

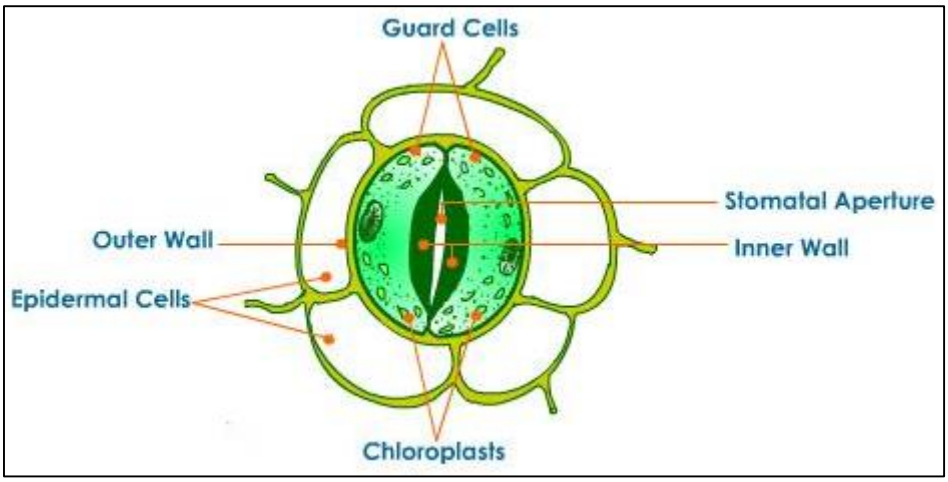

Figure 1 Stomatal structure (from www.suggestkeyword.com).

The walls of the guard cells are thickened on the side surrounding the pore only, giving them their characteristic shape. This unique thickening of the cell wall is what allows them to open and close the stomatal pore in response to changes in turgor pressure within the guard cells (Bidwell, 1974). The guard cells differ from ordinary epidermal cells further in that they contain chloroplasts. The basic stomatal structure is similar across species, but there are two differently shaped types of stomata – kidney-shaped (elliptical) and dumbbell-shaped (graminaceous) stomata (Martin *et al.*, 1983; Weyers & Meidner, 1990). Grasses and sedges (type of wetland plant) have graminaceous stomata while all other plant species, including *Vitis vinifera*, have elliptic stomata (Martin *et al.*, 1983). Stomata may also be raised or sunken as opposed to being level with the surrounding epidermal cells (Pratt, 1974) in an attempt to limit transpiration under conditions of water stress. It has also been found that the cuticle covering the epidermal and guard cells extends to the ventral and inner walls of the guard cells (Weyers & Meidner, 1990).

2.2.2 Development

2.2.2.1 Differentiation and specialisation

The processes involved in stomatal development have been well documented in *Arabidopsis thaliana*, especially, with molecular control mechanisms also identified (Casson & Hetherington, 2010; Pillitteri & Torii, 2012). From these studies it has been found that stomata develop from epidermal (protodermal) cells through a series of asymmetric and symmetric cell divisions yielding specialised guard cells as the end product. This series of divisions is known as the "stomatal lineage", and is diagrammatically presented in Figure 2.

Stellenbosch University https://scholar.sun.ac.za

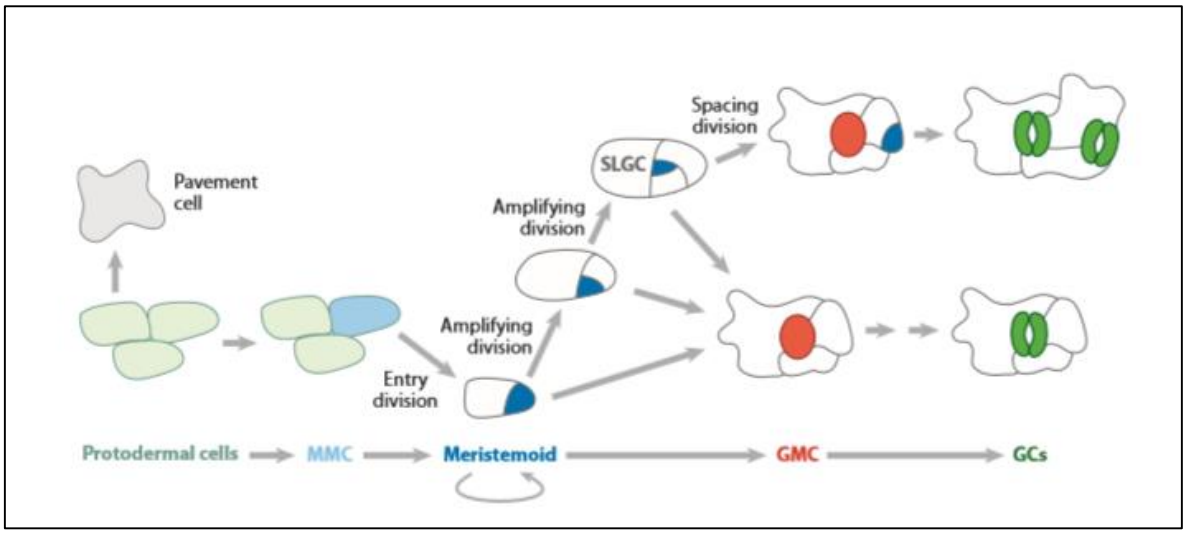

Figure 2 The stomatal lineage in *Arabidopsis thaliana* (Pillitteri & Torii, 2012).

Firstly, protodermal cells undergo a transition to a meristemoid mother cell (MMC), which then undergoes an asymmetrical division (termed the entry division) to yield a meristemoid and larger sister cell. The meristemoid can then either differentiate into a guard mother cell (GMC) or enter into further asymmetric divisions (amplifying divisions) yielding a satellite meristemoid and stomata lineage ground cell (SLGC) before differentiating. Satellite meristemoids, through spacing divisions, differentiate into GMC's. The guard mother cell then divides only once in a symmetrical fashion to produce two identical guard cells (GC's). The final stage of development is stomatal morphogenesis entailing the thickening of the inner guard-cell walls and the separation of the cells from one another to form the pore. Not all protodermal cells undergo this process and those which do not will become pavement cells. The SLGC's can also become pavement cells instead of following the stomatal lineage.

The differentiation of stomata has been found to be regulated by various genes, some of which are listed in Table 1 below. This is still a very complex area of study and not all mechanisms of control are equally understood. Very little studies have been conducted on a molecular level on grapevines. Plant hormones such as gibberellins, ethylene and auxin have also been shown to play a role in controlling the development of stomata. This will be discussed briefly in a later section of this chapter.

Stellenbosch University https://scholar.sun.ac.za

Table 1 Genes regulating stomatal differentiation in *Arabidopsis thaliana.*

Gene name	Symbol	Target	Action	Other possible targets and actions
SPEECHLESS	SPCH	Entry divisions	Promotes	Amplifying divisions - promotes
				Spacing divisions - promotes
MUTE	MUTE	Asymmetric divisions in M[1]	Terminates	Amplifying divisions - inhibits
FAMA	FAMA	Symmetrical divisions (GMC[2] to GC's[3])	Controls	Not applicable
FOUR LIPS	FLP	Symmetrical divisions (GMC to GC's)	Terminates	Not applicable
MYB88	MYB88	Symmetrical divisions (GMC to GC's)	Terminates	Not applicable

[1] Meristemoid
[2] Guard mother cell
[3] Guard cells

This developmental process may, however, differ between plants; e.g. it has been found that in mosses, stomata form from a single asymmetrical division yielding a guard mother cell that will divide either partially, to produce one guard cell, or completely, to give rise to two guard cells surrounding a pore (Payne, 1979). It may also be that other plants do not employ asymmetrical division and use symmetrical divisions instead. This documented mechanism could, however, serve as a guideline for plants in general until more detail is available for other species. With the classification of the grapevine genome, studies of a similar nature to that carried out on model plants should be possible in the near future.

2.2.2.2 Stomatal patterning and distribution

In general, a minimum of one epidermal cell will be found between stomata – this is known as the "one-cell spacing rule" (Peterson *et al.*, 2010; Rogiers *et al.*, 2011) and forms the basis for stomatal patterning. This rule is maintained through the correct orientation of spacing divisions which will ensure that new meristemoids do not form next to existing stoma or precursors. Cases have been found, however, where this has happened with two meristemoid mother cells developing alongside one another yielding two adjacent meristemoids upon division. This erroneous spacing could be corrected if one of the meristemoids either undergoes an orientated spacing division, thereby inserting a daughter cell between itself and the other meristemoid, or alternatively differentiating into a pavement cell (Geisler *et al.*, 2000; Casson & Gray, 2008). The correct patterning of stomata across the epidermis is necessary to ensure their efficiency, since stomatal movements rely largely on ion fluxes between themselves and the surrounding epidermal cells (Peterson *et al.*, 2010). However, stomatal clustering has been observed in certain plants growing under conditions of high temperature and limited water availability (Lehmann & Or, 2015) as a means of limiting transpiration.

There is some contradiction in literature regarding the nature of stomatal development and therefore also stomatal patterning. It is mostly documented that stomata form randomly within the epidermis (Rogiers *et al.*, 2011), but others state the opposite (Peterson *et al.*, 2010). Perhaps it would be more correct to state that stomatal development is not limited to selected epidermal cells and that it could in fact originate from any cell with a capacity to undergo division, but also that not

Stellenbosch University https://scholar.sun.ac.za

all cells with this ability will differentiate into stomata (Nadeau & Sack, 2002). With the one-cell spacing rule kept in mind, this randomisation is limited. Various environmental factors determine stomatal density and index which are defined as the number of stomata per unit leaf area and the number of stomata in relationship to total epidermal cells respectively. Stomata are therefore not expected to form closer than one cell apart, but are not limited to only one cell separating them, adding some degree of randomness.

Stomatal patterning is also controlled by numerous genes, a few of which are listed in Table 2. As with the genes controlling stomatal differentiation, there are still some uncertainties regarding the precise mechanisms by which the genes account for the control of the spatial arrangement of stomata. Patterning can be summarised as a result of the type, position and number of cell divisions that occur (Peterson *et al.*, 2010).

Table 2 Genes regulating stomatal patterning in *Arabidopsis thaliana.*

Gene name	Symbol	Target	Action
TOO MANY MOUTHS	TMM	Amplifying divisions	Regulate number and orientation
		Spacing divisions	Regulate number and orientation
ERECTA	ER	Entry divisions	Inhibits
		M[1] differentiation	Promotes
ERECTA-LIKE 1	ERL1	M differentiation	Inhibits
ERECTA-LIKE 2	ERL2	Amplifying divisions	Regulatory
STOMATAL DENSITY AND DISTRIBUTION 1	SDD1	Entry divisions	Regulate number
		Amplifying divisions	Regulate number
		Spacing divisions	Regulate orientation
MAPK - cascade		Stomatal development as a whole	Negatively regulate

[1] Meristemoid

The distribution of stomata across the leaf is of such a nature that water loss is minimised, and it varies greatly across species. Leaves can be amphistmomatous, with stomata occurring on both the adaxial (upper) and abaxial (lower) leaf surfaces – with more stomata usually occurring on the abaxial surface. In addition, leaves of some trees have been found to be hypostomatous with stomata only present on the abaxial leaf surface. Water-lilies in particular, have stomata occurring on the adaxial surface only and are termed epistomatous (Lawson, 2009). In *Vitis* stomata are almost entirely absent from the adaxial epidermis (Pratt, 1974; Düring, 1980). Concentration of stomata on the underside of the leaf reduces transpiration since this surface is generally cooler than the upper leaf surface, which is directly exposed to sunlight (Martin & Glover, 2007). Stomata are rarely found over main veins (Martin & Glover, 2007), since this would facilitate transpiration. This may be related to the fact that palisade mesophyll cells responsible for photosynthesis are rarely found close to vascular bundles (veins). This theory leads us to wonder whether there is a connection between stomatal distribution and underlying cell layers i.e. whether internal anatomy plays some part in stomatal patterning (Casson & Gray, 2008).

Stellenbosch University https://scholar.sun.ac.za

2.3 Stomatal function

As mentioned previously, stomata are concerned mainly with gaseous exchange, the main purpose being to optimise and regulate stomatal conductance of CO_2 and water vapour in order to balance photosynthesis with the amount of water available to the plant (Payne, 1979; Chaerle *et al.*, 2005; Casson & Gray, 2008). Stomata fulfil other functions as well, including the prevention of xylem embolism, nutrient and hormone transport and cooling of the leaves, but these most probably evolved over time (Raven, 2002).

2.3.1 Stomatal movement

Short-term responses to environmental stimuli are usually brought about by the opening or closing of the stomatal pore – this is referred to as stomatal movement. This opening and closing affects stomatal functioning. Environmental stimuli affecting stomatal functioning include light (quantity and quality), atmospheric CO_2 concentration, temperature, relative humidity and soil moisture content. Phytohormones can also play a role in guiding stomatal functioning (Kearns & Assmann, 1993).

The guard cells are responsible for bringing about the opening and closing of stomata – this occurs in response to an increase or decrease in guard cell turgor through osmoregulation. When the osmotic potential of guard cells increases, water is taken up from the surrounding epidermal cells. This increases the turgor pressure within the guard cells causing them to swell, ultimately opening the stomatal pore. The reverse occurs during stomatal closure. Many theories have been proposed for the increase in guard cell osmotic pressure including the uptake of K^+ and Cl^- ions, malate synthesis or sucrose accumulation (Assmann & Shimazaki, 1999; Roelfsema & Hedrich, 2005). It is proposed that the K^+ ion influx (counter balanced by the uptake of Cl^- and $malate^-$) drives the initial rapid opening and that the additional action of sucrose comes into play later in order to maintain the guard cell turgor (Roelfsema & Hedrich, 2005; Lawson, 2009). It has also been noted that the surrounding epidermal cells provide a backpressure which hampers guard cell swelling. It is thus proposed that the accumulation of sucrose acts as the additional osmoticum required to achieve a great enough guard cell turgor pressure to overcome this counter pressure (Roelfsema & Hedrich, 2005). Ion and solute efflux from the guard cells is responsible for bringing about stomatal closure. Both stomatal opening and closing have been found to be energy dependent processes. K^+ ion influx is driven by a H^+ gradient that is activated by proton ATPase (Lawson, 2009). It is thought that the rapid disappearance of sucrose from the guard cells upon stomatal closing is brought about by its extrusion from the guard cells (Roelfsema & Hedrich, 2005). A simplified diagram of stomatal opening and closing is shown in Figure 3.

Stellenbosch University https://scholar.sun.ac.za

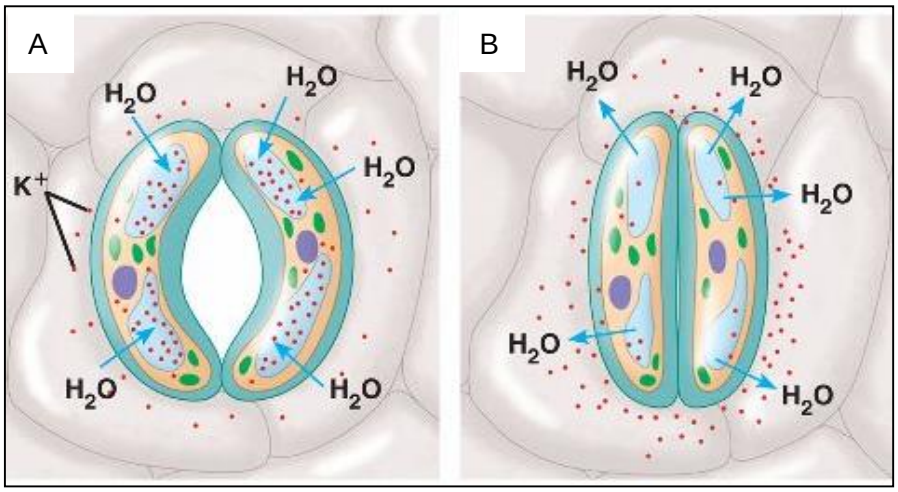

Figure 3 Basic osmoregulatory mechanism of A) stomatal opening and B) stomatal closing through K^+ ion influx and efflux (from www.wiki.bio.purdue.edu).

2.3.2 Function within the leaf

Gaseous exchange takes place primarily through the stomata with CO_2 moving into the leaf intercellular spaces while water vapour is simultaneously lost. It has, however, been found that some gaseous exchange can take place through the cuticle itself, but this is minimal and the barrier to CO_2 across this path is greater than for water vapour (Boyer *et al.*, 1997). The CO_2 taken up through the stomata, diffuse into the mesophyll cells where it is used as a substrate for photosynthesis. Stomata are thus directly involved in the plant's energy production process. Plants will always strive to optimise the amount of CO_2 gained per unit of water lost.

Photosynthesis occurs only in the presence of light and it is also important to note that light stimulates stomatal opening. This will be discussed in detail in a later section of this chapter. Photosynthesis is also temperature dependent with net photosynthesis being optimal at leaf temperatures of between 25°C and 30°C (Keller, 2010). During transpiration heat is lost in conjunction with the water vapour and this has a cooling effect on the leaves. It has also been proposed that the cooling effect of transpiration is of importance on a canopy scale where the convective boundary layer and not the stomata as such are responsible for controlling transpiration (Raven, 2002). This convective boundary layer is a thin film of still, moist air at the surface of the leaf, offering resistance to transpiration (Keller, 2010).

2.3.3 Function within the plant

Plants in which a cuticle, intercellular air spaces and an endohydric water conducting system are present, are able to regulate their degree of hydration under fluctuating soil moisture conditions and evaporative demand from the environment. This process is known as homoiohydry and stomata play an important role in it (Raven, 2002). Water conservation is usually achieved by the closure of the stomata and this obviously occurs at the expense of CO_2 uptake. Another aspect of stomatal functioning related to water conservation is the prevention of xylem embolism which would lead to a loss of xylem transport in affected vessels (Tyree & Sperry, 1988; Jones, 1998; Raven, 2002). This could lead to a negative impact on the overall plant water status.

When plants transpire, they lose water to the atmosphere. This causes a water pressure gradient throughout the plant which drives the uptake of water through roots from the soil and the transport thereof through the plant along with essential nutrients. It has been found, however, that the rate of transpiration has little effect on the amount of soil-derived nutrients that is delivered to the shoot as a whole. This can be explained by the fact that xylem loading in the root can account for more

Stellenbosch University https://scholar.sun.ac.za

nutrients being transported per unit of water (Raven, 2002). Hormones such as auxin, cytokinins and gibberellins, which are produced in the roots are also transported to shoots in the transpiration stream.

2.4 Factors affecting stomatal development and function

Plants need to adapt to unfavourable or changing environmental conditions, and the stomata are vital in achieving this (Casson & Gray, 2008). Gaseous exchange can be regulated by means of opening and closing the stomatal pore – this is a relatively short-term response and is reversed once conditions return to the original state). Under prevailing environmental conditions the strategy is to alter the number and nature of stomata formed in new organs i.e. altering the stomatal size and density on expanding leaves (Casson & Gray, 2008) – this is a long-term and more permanent response. In the latter case signals are sent to the developing leaves from mature leaves that perceive the environmental stimulus indicating the necessity for a change in stomatal density (Lake *et al.*, 2001). The exact mechanism of this signalling system is still unknown, with uncertainties regarding how the environmental stimuli are received, the kind of signals that arise from them and where exactly in the developmental process they bring about their effect to alter stomatal density (Casson & Gray, 2008). It must also be noted that many of the signalling mechanisms interact in such a way that the response to one signal may alter the guard cell's response to another signal (Roelfsema & Hedrich, 2005). The short-term responses of stomatal opening and closing are an example of an elastic physiological adaptation, while the long-term responses are representative of plastic responses to prolonged changes in environmental conditions.

2.4.1 Abiotic factors

2.4.1.1 Carbon dioxide concentration [CO_2]

A short-term response to a momentary increase in CO_2 concentration occurs when the stomata close partially in order to balance the uptake of CO_2 with transpiration. They will re-open when levels become ambient once more.

Many studies have been conducted to investigate the effect of elevated atmospheric CO_2 levels on stomatal density. This was also investigated over geological time using fossil records and correlating observations with periods of high atmospheric CO_2 (Franks & Beerling, 2009). The general trend was for stomatal density to decrease with an increase in CO_2 concentration (Casson & Gray, 2008; Franks & Beerling, 2009). The effect of elevated CO_2 levels on stomatal size was also investigated in the geological time study and it was found to increase. A correlation has been established between the stomatal conductance of mature leaves and young leaves in Poplar (Miyazawa *et al.*, 2006) supporting the theory that changes in stomatal density are signalled by mature leaves. Lake *et al.* (2001) did a similar investigation using *Arabidopsis thaliana* and made the conclusion that young expanding leaves are perhaps unable to perceive or respond to the change in CO_2. The effect of CO_2 on *Vitis vinifera* was studied by Rogiers *et al.* (2011), where CO_2 levels were lowered, with an increase in stomatal density also being confirmed. In molecular studies the gene *HIGH CARBON DIOXIDE* (*HIC*) has been implicated in effecting the decrease in stomatal density under conditions of elevated CO_2 (Gray *et al.*, 2000). The exact working of HIC respective to this is still not understood, but it appears to be involved in production of wax cuticle components (Casson & Hetherington, 2010).

Stellenbosch University https://scholar.sun.ac.za

2.4.1.2 Light

Diurnal alternation between stomatal opening and closing are short-term responses to day and night (increased and decreased light intensity). Stomatal functioning undergoes circadian rhythms in response to light. There are two types of rhythmicity involved in stomatal regulation (Gorton *et al.*, 1993):

1. The alternation between opening and closing that occurs over a 24 hour period (day-night responses).
2. The rhythm in the speed and degree of the response to light, which peaks every 24 hours – stomata open more rapidly in response to light during the "day phase" of the cycle and close more quickly in response to darkness during the "night phase".

Both blue and red light bring about stomatal opening. It is thus agreed that there are two photoreceptor systems involved (Mansfield *et al.*, 1981; Martin *et al.*, 1983; Zeiger & Zhu, 1998; Assmann & Shimazaki, 1999):

1. Photosynthetic active radiation (PAR)-dependent photosystem.
2. Blue-light specific photosystem.

The guard cell chloroplasts fulfil the role of the PAR sensitive system, but as of yet the blue-light receptor has not been identified (Gorton *et al.*, 1993; Assmann & Shimazaki, 1999). The fact that stomatal opening occurs in plant species lacking guard cell chloroplasts when subjected to blue light, confirms that a separate blue-light receptor must be involved (Zeiger *et al.*, 1985). This is further supported by the observation that low intensity blue light is more effective at stimulating stomatal opening than low intensity red light (Kearns & Assmann, 1993). Some suggestions about what this blue-light photoreceptor could be have been made, and these include carotenoids, such as zeaxanthin, and flavins (Zeiger & Zhu, 1998).

The blue-light receptor or system is most likely saturated for the greater part of the day and this indicates that it may be important in detecting daybreak (Zeiger *et al.*, 1981). The response of stomata to short pulses of blue light shows that the blue-light system is active under sunfleck light conditions as well (Zeiger & Field, 1982; Gorton *et al.*, 1993).

In most cases an increase in light intensity results in an increase in stomatal index (the number of stomata in relation to the total number of cells within an area) – mostly through an increase in stomatal number (Casson *et al.*, 2009). It is thus evident that an increase in light intensity positively affects stomatal cell fate. This effect is initiated through phytochrome photoreceptors and it has been determined that phyB is the main photoreceptor involved. PhyB works together with phytochrome interacting factor 4 (PIF4) to bring about the response (Casson *et al.*, 2009). In a study by Palliotti *et al.* (2000) the stomatal densities of shade and sun leaves in two grapevine cultivars, Cabernet franc and Trebbiano Toscano, were determined. The primary and lateral leaves of both cultivars showed an adaptation to shade – stomatal density decreased as would be expected from observations in other studies. Stomata were also found to be larger in the shaded leaves. Other changes in shaded leaves included an increase in leaf area and a decrease in the cuticular wax and abaxial trichomes (hairs). Since transpiration from shaded leaves is lower, there is no need to further limit transpiration through decreasing leaf size and producing high densities of trichomes.

2.4.1.3 Drought

Some plants grow in water scarce areas and have adapted to these conditions in various ways, including having sunken stomata, trichomes on the leaf surface and thick cuticular wax layers. All

Stellenbosch University https://scholar.sun.ac.za

of these adaptations are aimed at reducing water loss through transpiration. Most land plants will undergo water stress in varying degrees at some point, and in order to survive these conditions they must make changes. Responses to water stress can be short-term or long-term and these are summarised in Figure 4.

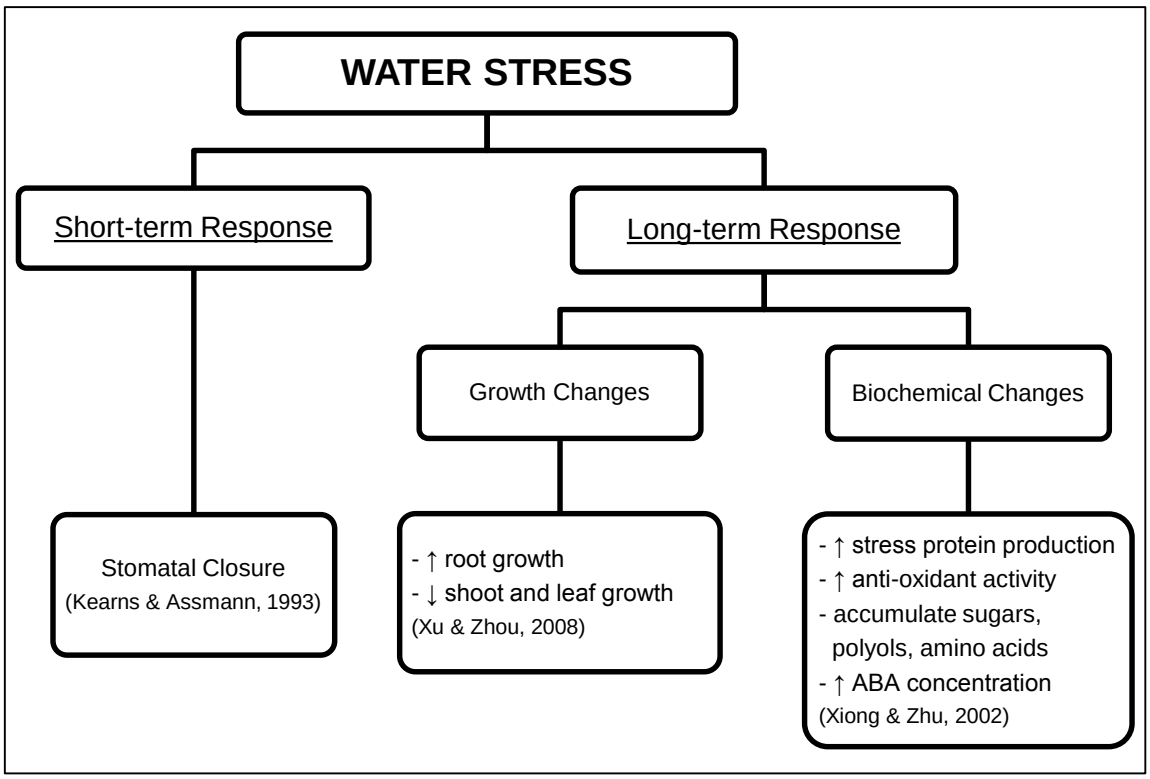

Figure 4 Schematic depiction of short- and long-term drought induced responses in plants (adapted from Arve *et al.* (2011)).

Responses to water stress are aimed at maximising water conservation. Stomatal closure is the first response to such conditions and will reverse once sufficient water is available again. Abscisic acid (ABA) production within the roots increases under conditions of limited water availability. It is transported to the leaves where it brings about stomatal closure by promoting K^+ efflux from the guard cells, which deceases their osmotic potential, in turn leading to water efflux (Kearns & Assmann, 1993).

The effect of water stress on stomatal development will depend upon the severity and duration of the stress (Xu & Zhou, 2008). Under moderate stress the stomatal number is increased, but it will decrease under severe water stress. Water stress also has an effect on stomatal size, with smaller stomata being observed for water-stressed plants. A general statement would be that water stress increases stomatal density while decreasing stomatal size. The reduction in stomatal size would allow for a quicker response to water stress in order to prevent water loss (Wang *et al.*, 2007).

The main purpose of stomatal adaptation (in both behaviour and development) in response to water deficit is to optimise the water use efficiency of the plant (Wang *et al.*, 2007). By limiting the amount of water lost through transpiration, the amount of CO_2 taken up for use in photosynthesis is also limited and there is thus a need for a compromise between the transpiration and photosynthetic rates to be established. This is done by altering stomatal behaviour, density and size (Wang *et al.*, 2007). The stomatal density has been found to be positively correlated with water use efficiency, since a higher stomatal density will increase the net assimilation of carbon to a greater degree than transpiration under moderate water stress (Xu & Zhou, 2008).

Stellenbosch University https://scholar.sun.ac.za

Growth and biochemical changes also occur with extended periods of water stress. With water stress the cells lose turgor pressure (excessive loss leads to wilting) and processes dependent on this, such as cell expansion, are therefore hindered (Arve *et al.*, 2011). This will lead to a reduction in plant growth (stems and leaves) which reduces the area from which transpiration can occur (Xu & Zhou, 2008). The root system, however, may be increased either laterally or by growing deeper in an attempt to increase the area over which water absorption is able to take place and to utilise water that is further away from the current root system. By increasing the production and accumulation of sugars, polyols and other solutes, the cell osmotic potential is lowered, allowing water absorption and the retention thereof. The stress hormone ABA is also produced under conditions of water stress, which affects the growth changes observed (Xiong & Zhu, 2002; Arve *et al.*, 2011). The way in which cells respond to drought stress with regard to their number and size, depends on the period of leaf growth during which water stress occurs (Xu & Zhou, 2008). In turn these responses may affect stomatal density and index as well.

2.4.1.4 Soil temperature

Rogiers *et al.* (2011) found that soil temperature also had an effect on stomatal density in *Vitis vinifera*. Plants growing in warmer soil displayed larger epidermal cells in the leaves, as well as a decrease in stomatal density. The opposite was true for plants grown in cooler soil. During this study a negative correlation was established between starch content of roots and trunks and stomatal density. The 'signalling of responses from mature leaves' theory (Lake *et al.*, 2001) for *Arabidopsis* is disputed for deciduous plants, such as the grapevine, since mature leaves are not present to effect changes in the first leaves for the season (Rogiers *et al.*, 2011). The environmental factors affecting stomatal density may therefore cause the changes via metabolic pathways related to carbohydrate reserves, including those involved in the diurnal regulation of starch stored in leaves (Rogiers *et al.*, 2011).

2.4.1.5 Relative humidity

The effect of relative humidity on stomatal development has been investigated in roses grown in greenhouses under high relative humidity (Torre *et al.*, 2003). In these studies the stomata were found to be large and non-functional, unable to close when plants were moved to conditions of lower humidity. High relative humidity also increased stomatal density in this study. Nejad & van Meeteren (2008) investigated the effect of relative humidity on *Tradescantia virginiana* and found that if plants initially grown under high relative humidity were moved to dryer conditions, expanding leaves were able to adapt and regain function of their stomata. This supports all other observations that there is some degree of plasticity involved in stomatal development and adaptation thereof – before leaves are completely developed they still have the ability to adjust according to stimuli and signals received.

2.4.2 Biotic factors

The plant itself also has an effect upon regulating stomatal functioning and development either because of its growth habit (vigour in particular) or internal signals (hormones). The biotic factors can however not be separated from abiotic effects completely, since the overall effect is usually brought about by a change in the latter.

2.4.2.1 Vigour and leaf size

It is difficult to separate vigour and leaf size since the latter is dependent on the former. With an increase in vigour, leaf size is also increased and this has various implications for stomatal size and density. Firstly, it may be that there is an increase in stomatal number due to the increase in

Stellenbosch University https://scholar.sun.ac.za

leaf size, but it may not be true that stomatal density is increased with leaf size. High vigour grapevines will have denser canopies due to this larger leaf size as well as an increase in the number of leaves produced. The increase in leaf number could be due to the presence of more shoots (both main and lateral) on vigorous vines. The more dense canopies may lead to shaded conditions, which may affect stomatal development indirectly. Under shaded conditions stomatal density would be expected to decrease while an increase in stomatal size should be noticed. A more vigorous growing vine has a greater water requirement and thus vigour may also bring about stomatal change (again indirectly) through water stress.

2.4.2.2 Leaf age

It is known that stomata develop on young, expanding leaves only. It is thus not possible for mature leaves to alter their stomatal density or size – instead a change is brought about in young leaves based on conditions experienced by the older leaves. The exact mechanisms by which these changes are brought about are still to be identified. This signalling mechanism by mature leaves may not be applicable to grapevine (Rogiers *et al.*, 2011). Furthermore, even though it is proposed that young leaves are perhaps unable to perceive or react to environmental factors (Lake *et al.*, 2001), this has not been definitively proven for all environmental stimuli.

2.4.2.3 Rootstock

Rootstocks can bring about various effects in the scion cultivars, including changes in vigour and increased drought resistance. The effects on scion water relations and vigour are usually closely linked (Jones, 2012). The latter may be brought about by more effective rootstock root systems allowing for better water utilisation, but possibly also through a change in stomatal development or functioning in the scion cultivar. Studies done on apples have found that stomatal size is decreased with the use of dwarfing rootstocks (Jones, 2012). In grapevines, it was found that the drought response changes in stomatal density and size for a particular scion cultivar differed when it was grafted onto different rootstocks (Serra *et al.*, 2014). The exact way in which rootstocks increase the drought tolerance of scions is still unknown, but it is most likely related to water uptake and transport, and the perception of drought stress and the resulting signalling to alter stomatal development and/or functioning (Serra *et al.*, 2014).

2.4.2.4 Plant hormones

Auxins have also been implicated in regulating stomatal opening and closing. Two methods of functioning have been identified depending on the type and concentration of auxin (Kearns & Assmann, 1993):

1. ATP-ase pump activation stimulation exceeds anion-channel activation and stomata open
2. Anion-channel activation is predominant and stomata close

ABA is a particular auxin closely involved in stomatal reactions to water stress, particularly stomatal closing. It has also been seen that ABA can lead to a decrease in stomatal density.

An increase in gibberellins will lead to an increase in stomatal density. The effects of auxin and gibberellin on stomata are closely linked since an increase in auxin concentration stimulates gibberellin activity (Casson & Gray, 2008).

Certain environmental conditions will also affect the hormonal impact on stomata. An increase in CO_2 concentration, for example, will increase the concentration of auxin, cytokinins and gibberellins in the leaves (Casson & Gray, 2008).

Stellenbosch University https://scholar.sun.ac.za

2.5 Methods used in stomatal research

In order to measure stomatal density and aperture one of two approaches can be followed. The first is measurements through microscopy (direct method), and the second estimates based on the measurement of stomatal conductance (indirect method) (Meidner, 1981). We will focus on the first approach here.

Microscopy can be conducted using standard light microscopy or scanning electron microscopy (SEM). Fresh leaf material (intact leaves or sections thereof) can be used in investigations employing both of these microscopy methods. In addition to this, epidermal peels or impressions can be used in light microscopy (Meidner, 1981; Weyers & Meidner, 1990). Figure 5 and Figure 6 show images obtained using SEM and light microscopy respectively.

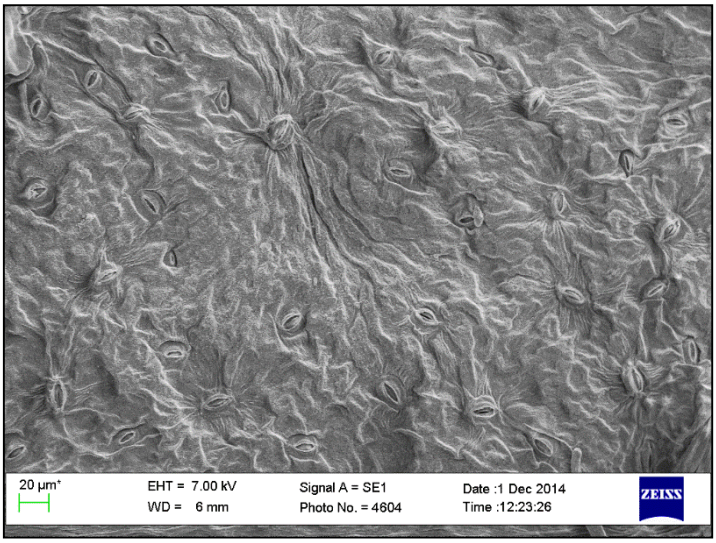

Figure 5 Scanning electron micrsocopy (SEM) image of a *Vitis vinifera* cv. Shiraz leaf at 400x magnification.

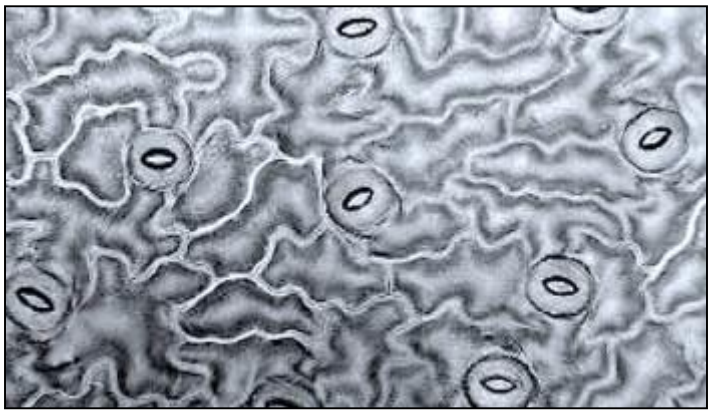

Figure 6 Stomata of *Acer rubrum* (red maple) viewed in a nail varnish impression at 400x magnification using light microscopy (http://www.esa.org/tiee/vol/v1/experiments/stomata/pdf/stomata.pdf © Marc Brodkin, 2000).

Using intact leaves for light microscopy may be challenging due to the light reflection of the cuticular wax cells which are differently orientated. Thick leaves may also limit the transmission of light through the sample (Meidner, 1981). Ways of overcoming these problems include using a light source from above (dissection microscope) or using a very strong light source from below. The latter is however not recommended when physiologically-related investigations are to be done, because both the light and heat can affect the stomata (Weyers & Meidner, 1990). Morphological investigations can be done quite successfully at a moderate level of transmitted light using 400x magnification or higher (Weyers & Meidner, 1990). In addition, it could be beneficial to submerge

Stellenbosch University https://scholar.sun.ac.za

the leaf or a cut section of the leaf in immersion oil. This limits the environmental effects on stomatal aperture (Meidner, 1981). Another way of optimising fresh leaf matter for light microscopy is to clear it of chlorophyll. Dow *et al.* (2013) used an ethanol-acetic acid solution to "bleach" the leaves. They were then softened in a potassium hydroxide solution and mounted directly on a slide for observation.

Direct epidermal peels may be taken from leaves by separating this layer from the underlying cell layers with forceps, but this is not easily achieved with all plant species. These direct peels may also be stained in order to facilitate the identification of guards cells – an example of such a process is the staining of guard cell starch granules using iodine-potassium-iodide (Düring, 1980). The use of impressions made from the epidermis is very popular and there are different ways in which these impressions can be made (Meidner, 1981; Weyers & Meidner, 1990). The most common method is applying a thin layer of clear nail varnish to the epidermis and peeling it off using clear adhesive tape or forceps once it has dried. Alternatively dental resin or a silicone rubber compound can be applied to the leaf and allowed to set making a mould of the leaf surface. Nail varnish or epoxy can then be used to fill the mould, creating a cast which can be examined under a microscope (Weyers & Meidner, 1990; Geisler *et al.*, 2000; Doheny-Adams *et al.*, 2012). When creating peels, it is however possible for the epidermis or imprint to become stretched when removing them from the leaf and this may affect stomatal aperture measurements. SEM work – which uses fresh leaf material – is thus very well suited for this type of measurement due to the great level of detail obtained, as well as the fact that there is no stretching of the material involved (Weyers & Meidner, 1990).Some advantages and disadvantages of the two microscopy methods are listed in Table 3.

Table 3 Advantages and disadvantages of light microscopy and scanning electron microscopy (SEM) for conducting stomatal investigations.

LIGHT MICROSCOPY		**SCANNING ELECTRON MICROSCOPY (SEM)**	
Advantages	Disadvantages	Advantages	Disadvantages
• Quick (depending on sample and preparation) • Simple • Stomatal counts • Stomatal measurements	• Thick leaves may cause problems • Peels can stretch	• Detailed images • Very accurate measuring • Relatively quick • No need to create peels/impressions	• Expensive equipment • Requires some skill

Another area of research is that which investigates the role of stomata in regulating transpiration and photosynthesis by investigating stomatal movement (Weyers & Meidner, 1990). Methods employed for such studies include gravimetric techniques (lysimetry), potometry, porometry and the determination of stomatal aperture *in vivo* (Weyers & Meidner, 1990). Lysimetry in this sense is very similar to soil lysimetry, except that the block of soil isolated contains a plant. This unit is then weighed over time to determine plant water loss and therefore, indirectly, stomatal action. Potometry measures the rate and amount of liquid that flows into a plant, wooded cutting or detached leaf (micro-potometry). Porometers measure the rate of air flow through the leaf blade and the first documented use was by Darwin and Pentz in 1911. There are then also those studies which look at the molecular mechanisms controlling stomatal responses. In such cases, controlled experiments are conducted, usually *in vitro* (Weyers & Meidner, 1990).These experiments are conducted on isolated sections of the plant and under controlled conditions and these isolates

Stellenbosch University https://scholar.sun.ac.za

include leaf discs, epidermal strips, protoplasts and subcellular fractions (Weyers & Meidner, 1990). The methods of stomatal research and the outcomes of each are summarised in Table 4.

Table 4 Methods used in stomatal research, the suitable materials used for each and the appropriate variables for measurements (the variables in parentheses are determined indirectly by calculation) [taken from Weyers and Meidner (1990)].

Method	**Suitable material**	**Appropriate variables**
Gravimetric determinations e.g. lysimetry	• plant community • single plant • excised shoot or leaf	• transpiration rate (leaf conductance)
Potometry	• single plant • excised shoot or leaf	• water uptake rate (transpiration rate, leaf conductance)
Porometry (either diffusion or mass –flow)	• attached or detached leaf • subsection of leaf	• stomatal or leaf conductance (rarely whole plant)
Determination of aperture in vivo	• subset of pores on a leaf	• mean aperture (stomatal conductance)
Leaf discs	• subsection of leaf	• mean aperture • stomatal or leaf conductance
Epidermal strips	• subset of cells on a leaf • subset of guard cells on a leaf	• mean aperture • tissue or cell solute content and biochemical variables
Protoplasts	• population of guard cells (often from several leaves)	• cell volume • cell solute content and biochemical variables
Individual guard cells	• individual guard cells	• cell solute content and biochemical variables
Subcellular fractions	• components of guard cells	• subcellular solute content, • subcellular biochemical variables

2.6 Conclusion

It is clear that stomata are necessary for plants to adapt to their environment in order to survive. Most of the adaptations are concerned with regulation of stomatal pore movement and stomatal density. Stomatal size can also play a role, but has not been studied as extensively. Serra (2014) found that severe water stress led to a decrease in stomatal size for Pinotage. He also found that there was an interaction effect of rootstock and water deficit, and water deficit and sun exposure on stomatal size. Stomata are mostly concerned with maintaining the water use efficiency of plants and it is therefore inevitable that changes in stomatal functioning and development will be brought about under different water regimes.

The environment acts as a stimulus to plants that effect changes in stomatal development and function. Carbon dioxide levels and light intensity have been the most studied environmental factors. There is, however, still a void in the studies particular to *Vitis vinifera*.

Stomatal density has been found to vary between cultivars of *Vitis vinifera* (Rogiers *et al.*, 2009) and a detailed investigation of this could help in establishing cultivar suitability for certain climates and conditions. With the changing global climate and the ability of plants to adapt to this, long-term studies on stomatal development and patterning can also shed some light on how certain cultivars would thrive or decline under future conditions.

There are numerous methods of investigating stomatal density and size – either by direct microscopy of leaves or impressions, or through estimations made from physiological

Stellenbosch University https://scholar.sun.ac.za

measurements. The method used must be suited to the information which is to be obtained from the study – certain methods may affect physiological functioning of stomata or their morphology.

2.7 Literature cited

Arve, L.E., Torre, S., Olsen, J.E. & Tanino, K.K., 2011. Stomatal Responses to Drought Stress and Air Humidity, Abiotic Stress in Plants - Mechanisms and Adaptations. Shanker, A. (ed). InTech.

Assmann, S.M. & Shimazaki, K-I., 1999. The multisensory guard cell. Stomatal responses to blue light and abscisic acid. Plant Physiol. 119(3), 809–815.

Bidwell, R.G.S., 1974. Plant Physiology. Macmillan, New York.

Boyer, J.S., Wong, S.C. & Farquhar, G.D., 1997. Carbon dioxide and water vapor exchange across leaf cuticle (epidermis) at various water potentials. Plant Physiol. 114(1), 185–191.

Casson, S.A., Franklin, K.A., Gray, J.E., Grierson, C.S., Whitelam, G.C. & Hetherington, A.M., 2009. Phytochrome B and PIF4 regulate stomatal development in response to light quantity. Curr. Biol. 19(3), 229–234.

Casson, S.A. & Gray, J.E., 2008. Influence of environmental factors on stomatal development. New Phytol. 178(1), 9–23.

Casson, S.A. & Hetherington, A.M., 2010. Environmental regulation of stomatal development. Curr. Opin. Plant Biol. 13(1), 90–95.

Chaerle, L., Saibo, N. & Van Der Straeten, D., 2005. Tuning the pores: towards engineering plants for improved water use efficiency. Trends Biotechnol. 23(6), 308–315.

Doheny-Adams, T., Hunt, L., Franks, P.J., Beerling, D.J. & Gray, J.E., 2012. Genetic manipulation of stomatal density influences stomatal size, plant growth and tolerance to restricted water supply across a growth carbon dioxide gradient. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 367(1588), 547–555.

Dow, G.J., Berry, J.A. & Bergmann, D.C., 2013. The physiological importance of developmental mechanisms that enforce proper stomatal spacing in *Arabidopsis thaliana*. New Phytol. , 1205–1217.

Düring, H., 1980. Stomatafrequenz bei Blättern von *Vitis*-Arten und -Sorten. Vitis 19, 91–98.

Franks, P.J. & Beerling, D.J., 2009. Maximum leaf conductance driven by carbon dioxide effects on stomatal size and density over geologic time. Proc. Natl. Acad. Sci. U. S. A. 106(25), 10343–10347.

Geisler, M., Nadeau, J.A. & Sack, F.D., 2000. Oriented asymmetric divisions that generate the stomatal spacing pattern in *Arabidopsis* are disrupted by the too many mouths mutation. Plant Cell 12(11), 2075–2086.

Gorton, H.L., Williams, W.E. & Assmann, S.M., 1993. Circadian rhythms in stomatal responsiveness to red and blue light. Plant Physiol. 103(2), 399–406.

Gray, J.E., Holroyd, G.H., van der Lee, F.M., Bahrami, A.R., Sijmons, P.C., Woodward, F.I., Schuch, W. & Hetherington, A.M., 2000. The HIC signalling pathway links carbon dioxide perception to stomatal development. Nature 408(6813), 713–716.

Hetherington, A.M. & Woodward, F.I., 2003. The role of stomata in sensing and driving enviromnetal change. Nature 424(6951), 901–908.

Jones, H.G., 1998. Stomatal control of photosynthesis and transpiration. J. Exp. Bot. 49(Special Issue), 387–398.

Jones, H.G., 2012. How do rootstocks control shoot water relations? New Phytol. 194(2), 301–303.

Kearns, E. V. & Assmann, S.M., 1993. The guard cell-environment connection. Plant Physiol. 102(3), 711–715.

Stellenbosch University https://scholar.sun.ac.za

Keller, M., 2010. The Science of Grapevines: Anatomy and Physiology. Academic Press, Amsterdam.

Kramer, P.J. & Boyer, J.S., 1995. Water Relations of Plants and Soils. Academic Press, San Diego.

Lake, J.A., Quick, W.P., Beerling, D.J. & Woodward, F.I., 2001. Signals from mature to new leaves. Nature 411(6834), 154.

Lawson, T., 2009. Guard cell photosynthesis and stomatal function. New Phytol. 181(1), 13–34.

Lehmann, P. & Or, D., 2015. Effects of stomata clustering on leaf gas exchange. New Phytol. 207, 1015–1025.

Mansfield, T.A., Travis, A.J. & Jarvis, R.G., 1981. Response to light and carbon dioxide. In: Jarvis, P.G. & Mansfield, T.A. (eds). Stomatal physiology. Cambridge University Press, Cambridge. pp. 119–136.

Martin, C. & Glover, B.J., 2007. Functional aspects of cell patterning in aerial epidermis. Curr. Opin. Plant Biol. 10(1), 70–82.

Martin, E.S., Donkin, M.E. & Stevens, R.A., 1983. Stomata. Edward Arnold (Publishers) Ltd., London.

Meidner, H., 1981. Measurements of stomatal aperture and responses to stimuli. In: Jarvis, P.G. & Mansfield, T.A. (eds). Stomatal physiology. Cambridge University Press, Cambridge. pp. 25–49.

Miyazawa, S-I., Livingston, N.J. & Turpin, D.H., 2006. Stomatal development in new leaves is related to the stomatal conductance of mature leaves in poplar (*Populus trichocarpa* x *P. deltoides*). J. Exp. Bot. 57(2), 373–380.

Nadeau, J.A. & Sack, F.D., 2002. Control of stomatal distribution on the *Arabidopsis* leaf surface. Science 296(5573), 1697–1700.

Nejad, A.R. & van Meeteren, U., 2008. Dynamics of adaptation of stomatal behaviour to moderate or high relative air humidity in *Tradescantia virginiana*. J. Exp. Bot. 59(2), 289–301.

Palliotti, A., Cartechini, A. & Ferranti, F., 2000. Morpho-anatomical and physiological characteristics of primary and lateral shoot leaves of Cabernet Franc and Trebbiano Toscano grapevines under two irradiance regimes. Am. J. Enol. Vitic. 51(2), 122–130.

Payne, W.W., 1979. Stomatal patterns in embryophytes: their evolution, ontogeny and interpretation. Taxon 28(1), 117–132.

Peterson, K.M., Rychel, A.L. & Torii, K.U., 2010. Out of the mouths of plants: the molecular basis of the evolution and diversity of stomatal development. Plant Cell 22(2), 296–306.

Pillitteri, L.J. & Torii, K.U., 2012. Mechanisms of stomatal development. Annu. Rev. Plant Biol. 63, 591–614.

Pratt, C., 1974. Vegetative anatomy of cultivated grapes - a review. Am. J. Enol. Vitic. 25(3), 131–150.

Raven, J.A., 2002. Selection pressures on stomatal evolution. New Phytol. 153(3), 371–386.

Roelfsema, M.R.G. & Hedrich, R., 2005. In the light of stomatal opening: new insights into “the watergate”. New Phytol. 167(3), 665–691.

Rogiers, S.Y., Greer, D.H., Hutton, R.J. & Landsberg, J.J., 2009. Does night-time transpiration contribute to anisohydric behaviour in a *Vitis vinifera* cultivar? J. Exp. Bot. 60(13), 3751–3763.

Rogiers, S.Y., Hardie, W.J. & Smith, J.P., 2011. Stomatal density of grapevine leaves (*Vitis vinifera* L.) responds to soil temperature and atmospheric carbon dioxide. Aust. J. Grape Wine Res. 17(2), 147–152.

Serra, I., 2014. Grapevine (*Vitis vinifera* L., cv. Pinotage) responses to water deficit modulated by rootstocks. PhD Dissertation, Stellenbosch University, Private Bag X1, 7602, Matieland (Stellenbosch), South Africa.

Stellenbosch University https://scholar.sun.ac.za

Serra, I., Strever, A., Myburgh, P.A. & Deloire, A., 2014. Review: the interaction between rootstocks and cultivars (*Vitis vinifera* L.) to enhance drought tolerance in grapevine. Aust. J. Grape Wine Res. 20(1), 1–14.

Torre, S., Fjeld, T., Gislerød, H.R. & Moe, R., 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. J. Am. Soc. Hortic. Sci. 128(4), 598–602.

Tyree, M.T. & Sperry, J.S., 1988. Do woody plants operate near the point of catastrophic xylem dysfunction caused by dynamic water stress?: answers from a model. Plant Physiol. 88(3), 574–580.

Wang, Y., Chen, X. & Xiang, C.-B., 2007. Stomatal density and bio-water saving. J. Integr. Plant Biol. 49(10), 1435–1444.

Weyers, J.D.B. & Meidner, H., 1990. Methods in Stomatal Research. Longman Scientific & Technical, England.

Xiong, L. & Zhu, J-K., 2002. Molecular and genetic aspects of plant responses to osmotic stress. Plant. Cell Environ. 25(2), 131–139.

Xu, Z. & Zhou, G., 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. J. Exp. Bot. 59(12), 3317–3325.

Zeiger, E., Field, C. & Mooney, H.A., 1981. Stomatal opening at dawn: possible roles of the blue-light response in nature. In: Smith, H. (ed). Plants and the Daylight Spectrum. Academic Press, London. pp. 391–407.

Zeiger, E. & Field, C., 1982. Photocontrol of the functional coupling between photosynthesis and stomatal conductance in the intact leaf: blue light and Par-dependent photosystems in guard cells. Plant Physiol. 70(2), 370–375.

Zeiger, E., Grivet, C., Assmann, S.M., Deitzer, G.F. & Hannegan, M.W., 1985. Stomatal limitation to carbon gain in *Paphiopedilum* sp. (Orchidaceae) and its reversal by blue light. Plant Physiol. 77(2), 456–460.

Zeiger, E. & Zhu, J., 1998. Role of zeaxanthin in blue light photoreception and the modulation of light-carbon dioxide interactions in guard cells. J. Exp. Bot. 49(Special Issue), 433–442.

Stellenbosch University https://scholar.sun.ac.za

Chapter 3

Methodology

Experimental layout and the use of field microscopy to investigate stomatal density non-destructively

Stellenbosch University https://scholar.sun.ac.za

CHAPTER III: EXPERIMENTAL LAYOUT AND THE USE OF FIELD MICROSCOPY TO INVESTIGATE STOMATAL DENSITY NON-DESTRUCTIVELY

3.1 Introduction

Stomata have been the focus of many studies. The method of research followed for a particular study depends on the desired objective of that study. The distribution of stomata across the leaf surface is often also investigated in conjunction with stomatal aperture. The aperture of stomata can also be used to determine stomatal conductance by using developed mathematical equations (Weyers & Meidner, 1990), thus providing an indirect means of determining stomatal function. Stomatal aperture and density are mostly determined through microscopic investigation, which can be conducted on intact leaves, leaf pieces or epidermal strips (either direct peels or impressions). Due to the destructive nature, it is not possible to measure the same sample more than once using these techniques (except when dental resin or silicon rubber is used to produce a mould of the leaf surface). The process of creating epidermal peels may lead to the distortion of epidermal cells and the stomatal guard cells due to a variety of reasons (Meidner, 1981; Weyers & Meidner, 1990). This is very important to keep in mind when determining stomatal aperture, but less of a problem if only stomatal density is of interest. In addition to light microscopy, scanning electron microscopy (SEM) is also used with great success and the latter is especially well suited to measuring stomatal aperture.

It appears that there has been little progress in the field of using intact leaves for long-term repeated studies to determine stomatal density. If such advances have been made, it has not been published widely. In this study an attempt was made at establishing a non-destructive method of doing stomatal investigations through the use of what we have termed “field microscopy”. This would make it possible to investigate the same leaves over a period of time. Measurements done on intact leaves that are still attached to the plant are mentioned in Meidner (1981), but most studies documented nowadays make use of epidermal peels or impressions (Doheny-Adams *et al.*, 2012), or scanning electron microscopy (Serra *et al.*, 2014).

3.2 Materials

3.2.1 Vineyard and cultivars chosen

The vineyard in which the experiment was conducted is a cultivar collection block at the Welgevallen experimental farm of the University of Stellenbosch (Stellenbosch, South Africa). More details about the block are given in Table 5. The block consists of 80 cultivars, each represented by three vines planted adjacent to one another in the same row. The block was originally established as a Chenin Blanc vineyard, after which the vines were top grafted to the current cultivars over a number of years. Four *Vitis vinifera* cultivars were selected for the study in order to determine whether there was variability in stomatal density and stomatal number per leaf between grapevine cultivars. The cultivars chosen were Cabernet Sauvignon, Grenache noir, Pinotage and Shiraz. These specific cultivars were selected for their difference in drought response – Shiraz is known to exhibit near-anisohydric behaviour under water stress conditions, while Cabernet Sauvignon and Grenache noir are near-isohydric (Schultz, 2003; Soar *et al.*, 2006; Tramontini *et al.*, 2014; Gerzon *et al.*, 2015). Isohydric and anisohydric refer to a plant’s ability or inability to regulate its water use respectively. Isohydric plants maintain a constant

Stellenbosch University https://scholar.sun.ac.za

midday stem water potential through regulating stomatal conductance while anisohydric plants do not (Sade *et al.*, 2012). The classification for Pinotage regarding its reaction to drought stress has not formally been made, but it does fairly well under rain-fed and bush vine cultivation, pointing towards a near-isohydric nature. Figure 7 and Figure 8 show the position of the vineyard block, and of the vines representing each of the cultivars chosen for the study within the block respectively.

Figure 7 Location of the vineyard at Welgevallen experimental farm, Stellenbosch.

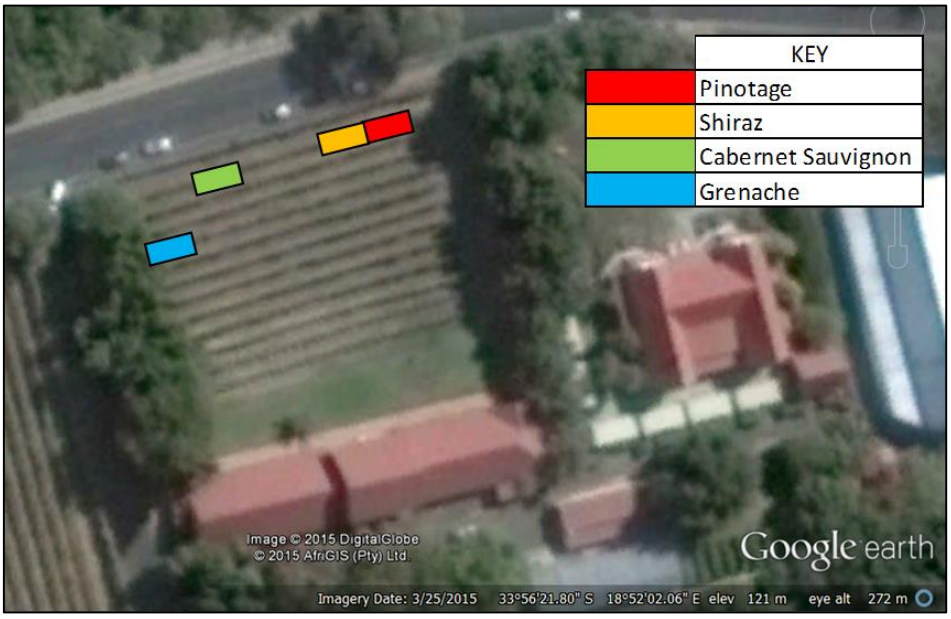

Figure 8 Indication of where the vines of each of the selected cultivars occur within the block.

Stellenbosch University https://scholar.sun.ac.za

Table 5 Particulars of the vineyard on Welgevallen experimental farm in which the experiment was conducted.

Descriptor	Welgevallen experimental farm – cultivar collection block
Year established	1991
Grapevine species	*Vitis vinifera*
Original scion cultivar	Chenin blanc
Rootstock	Richter 99 (*Vitis Berlandieri* x *Vitis rupestris*)
Top grafted cultivars selected for the study	Pinotage, Shiraz, Cabernet Sauvignon, Grenache noir
Year(s) top grafted	Pinotage, Shiraz & Cabernet Sauvignon: 1993/94 Grenache noir: 1995/96
Row orientation	East North East – West South West
Terrain	Flat
Lat/Long/elevation	33°56'22.37''S 18°51'58.49''E 121m
Vine spacing	2.75 m x 1.40 m
Trellis/training system	7-wire hedge trellis system with three sets of moveable foliage wires
Irrigation system	Rain-fed
Pruning system	Spur

3.2.2 Leaf selection

Another objective of the study was to determine whether stomatal density and stomatal number per leaf differ between leaves occurring at different positions on a shoot. In order to determine this, leaves were selected to represent Basal, Middle and Apical positions on a shoot according to the node position at which they occurred - Table 6 shows the classification used for this. The leaves were selected on 25 November 2014 (after flowering).

Table 6 Node ranges for classification of Basal, Middle and Apical leaves

Leaf position descriptor	Node range
Basal	≤ 9
Middle	9 – 13
Apical	≥ 13

Two leaves representing each leaf position were selected for each of the cultivars. Thus, each cultivar had two sets of Basal, Middle and Apical leaves. The one set of leaves occurred on one shoot and the other on a different shoot on a different vine. As the season progressed and the shoots grew, additional apical leaves were selected on the shoots already bearing the selected Basal, Middle and Apical leaves. These additional leaves were termed "Apical 2" and "Apical 3" and were selected on 12 December 2014 and 13 January 2015 respectively. Apical 2 leaves were positioned five nodes up from the Apical leaves and Apical 3 leaves again occurred five nodes above the Apical 2 leaves. Additional leaves ("General leaves") were also selected for each cultivar and this was done earlier in the season (9 November 2014). These leaves were all of similar age and position on the shoots. Data obtained from these leaves were used in the

Stellenbosch University https://scholar.sun.ac.za

image-analysis method comparison process (which will be discussed later in this chapter) and not for the main study described in Chapter 4.

The shoots on which the selected leaves occurred were marked using plastic strips on which the field repeat (leaf) number was indicated. The leaves were identified by tagging the shoot around the internode below the leaves with tape. The leaf position and field repeat number was written on this tape as well using a permanent marker pen. Figure 9 and Figure 10 show the methods used to mark the shoots and leaves for the easy identification of the selected leaves. The selection dates of the leaves are represented in the form of a timeline in Figure 11.

Figure 9 Example of coloured plastic strips used for marking shoots that have leaves selected for observations on them.

Figure 10 A selected leaf with tape marking the internode below it for identification.

Stellenbosch University https://scholar.sun.ac.za

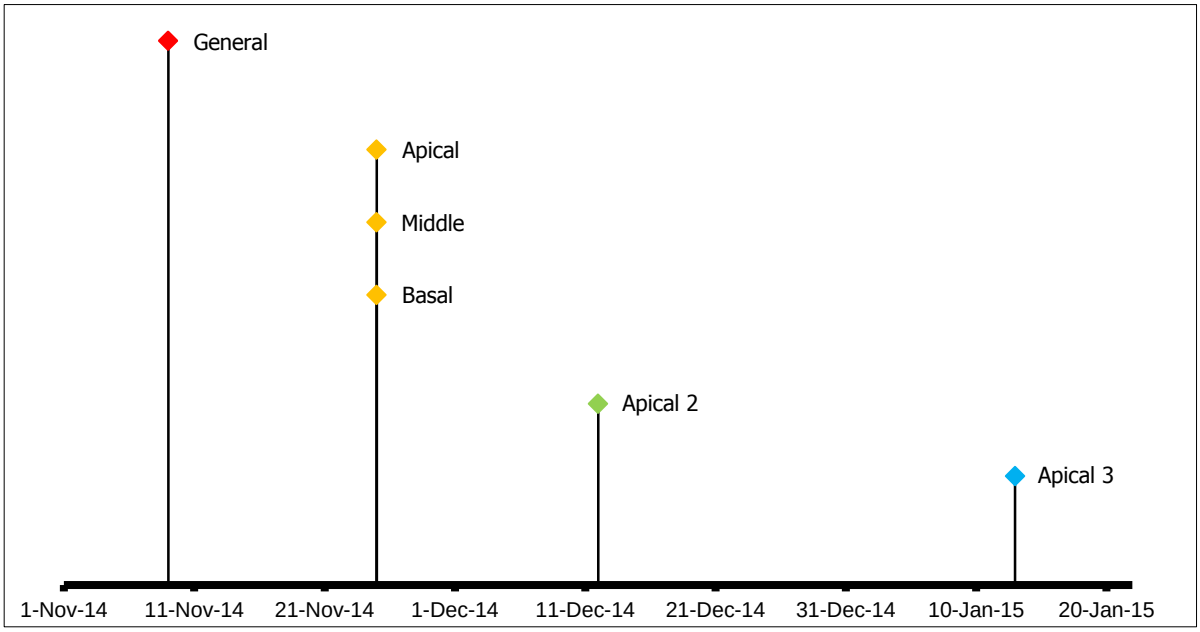

Figure 11 Timeline for the selection dates of leaves for the study.

3.2.3 Equipment used for field microscopy

The specific portable digital microscope used was the ProScope Mobile (Bodelin Technologies, Lake Oswego, Oregon, USA) which is used in conjunction with an iPad™ mini (Apple Inc., Cupertino, California, USA). The microscope connects to the iPad™ wirelessly and makes use of the specially developed application, AirMicroPad (Scalar Corporation, Tokyo, Japan), to display a live-feed view of the area investigated on the screen. Images can then be captured from this live-feed as photographs by touching the “live-view capture” icon on the screen. Alternatively, the capture button on the microscope can also be used. Through the capturing process the photographs are saved on the iPad™ and can later be downloaded to a computer. The parts of the microscope are identified in Figure 12.

Stellenbosch University https://scholar.sun.ac.za

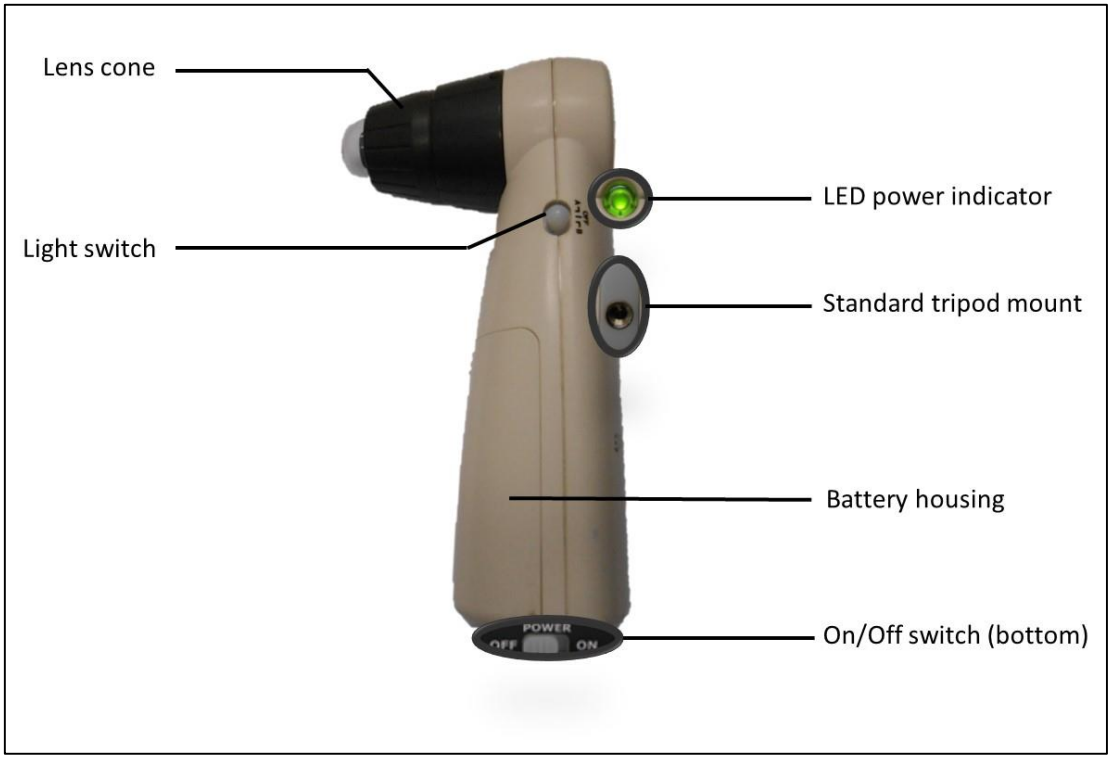

Figure 12 Portable microscope (side view) with parts indicated (adapted from www.bodelin.com).

The microscope works in the same way as a dissection microscope, where the light source illuminates the sample from above and the sample is thus viewed under reflected (episcopic) light and not transmitted (diascopic) light (Nothnagle *et al.*, 2015). The light source for this microscope consists of six white light emitting diodes (LED's) which are housed around the lens (Figure 13).

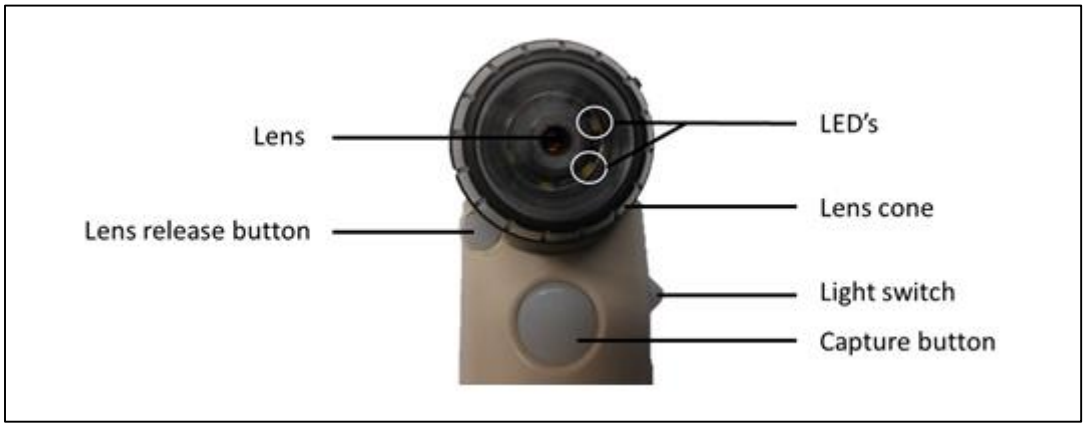

Figure 13 Portable microscope (view from underside) showing the LED's housed around the lens and other parts.

The microscope comes standard fitted with a 50x magnification lens, but since the reported size of grapevine stomata are between 12.36 µm and 20.31 µm (Palliotti *et al.*, 2000; Serra *et al.*, 2014) a magnification of 50x will be insufficient to be able to see and count stomata effectively. In this study, a 400x magnification lens was therefore used with the microscope. The microscope has a maximum imaging resolution of two megapixels (1200 x 1600 pixels), and video capabilities of VGA (video graphics array) resolution (640 x 480 pixels).

Stellenbosch University https://scholar.sun.ac.za

3.3 Methods

3.3.1 Vegetative measurements

3.3.1.1 Main central vein (L1) length

The L1 (main central vein) length of the leaves was measured in millimetres (mm). This was done periodically from the time of selection, until no further increase in length was observed between successive measurements.

3.3.1.2 Leaf area and correlation with main central vein (L1) length

By drawing up a correlation curve between the L1 length of a leaf and its measured leaf area (cm^2), the formula derived from this curve can be used to determine the area of a leaf non-destructively if the L1 length is known. Such correlation graphs were drawn up for each of the cultivars. For this it was necessary to obtain data of known L1 lengths and corresponding leaf areas – three representative shoots were selected at the end of the season for each cultivar and the L1 length and area for each of the leaves on those shoots measured. Leaves from each shoot were removed in order from the base of the shoot to the tip, and the L1 length of each determined in that order by measuring along the L1 vein using an ordinary material measuring tape. Using this same order, the leaves were fed through an electronic leaf surface area meter (Delta-T Devices, Cambridge, United Kingdom) and the areas recorded in square centimetres (cm^2). By measuring the L1 length and leaf area in the same order, the two measurements can be correlated.

3.3.2 Field microscopy

3.3.2.1 Imaging procedure

Images were taken at six different positions on the underside of the selected leaves - the distribution of these observation positions across the leaf surface is shown in Figure 14. This added another dynamic to the study, namely the investigation of stomatal density over different, but standardised, areas on the same leaf. The underside was chosen for the study since grapevine leaves are hypostomatous with most stomata occurring on the abaxial leaf surface (Pratt, 1974; Düring, 1980). The spatial orientation of the six positions were selected in order to include two leaf lobes and areas close to the vein, in the middle of the lobe and near the leaf margin.

Stellenbosch University https://scholar.sun.ac.za

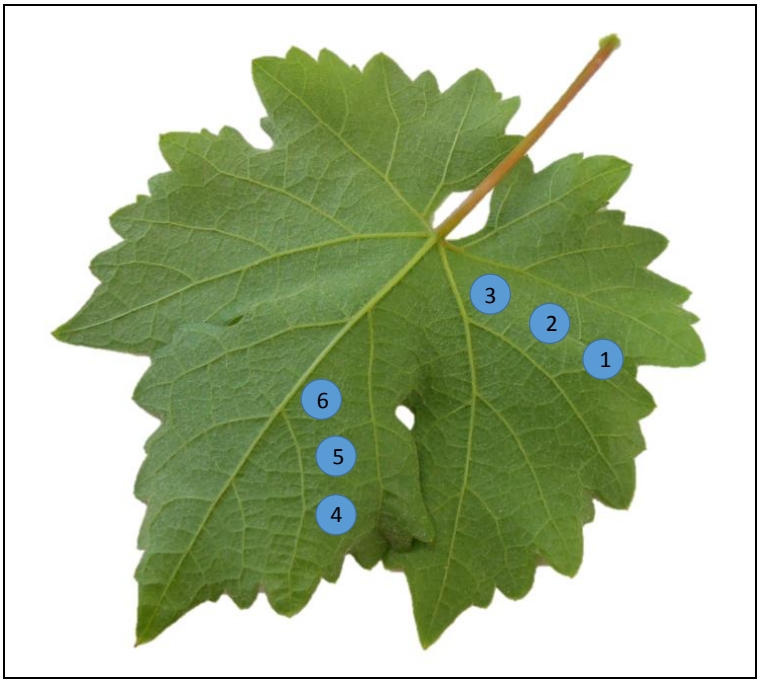

Figure 14 Distribution of the observation positions over the leaf surface (abaxial).

The imaging process was started by first removing the hairs (trichomes) on the underside of the leaves without causing damage to the leaf surface. Masking tape was used effectively for this by repeatedly dabbing the sticky side of the tape on the same spot on the leaf until the hair was removed. It should, however, be mentioned that some stray hairs occasionally remained on the leaf surface, which could interfere with imaging. Interesting to note is that Grenache noir leaves had no hairs on the abaxial leaf surface, and this hair-removal step was thus not required.

With the light source on, the microscope was placed on the leaf surface at the position to be investigated with the plastic dome touching the leaf surface. The microscope was held in one hand by gripping the “head” of the microscope between the thumb and index finger with the thumb on the back of the microscope and the index finger pushing the leaf against the dome. The image was brought into focus by turning the lens cone, which in turn adjusted the microscope’s working distance. Thus the focussing of the digital microscope works much in the same way as that of a conventional light microscope, where the stage height is adjusted altering the working distance and bringing an image into focus. The ambient light conditions interfered with image quality – this was overcome to a large extent by shading the investigated leaf with an umbrella and cupping one’s free hand around the lens cone creating a darker surrounding. This allowed for the illumination of the investigated area to be primarily due to the microscope’s light source giving a clearer image on the iPad™. The ambient light intensity is very high during the middle of the day and major imaging problems were experienced between 11 am and 2 pm. This was due to the fact that the cupping of one’s hand around the lens cone was not sufficient in providing a darker environment at this time. Investigations during this time of day were therefore avoided as far as possible.

The capture button on the microscope body was not used, since it often led to slight movement of the microscope when being pressed, thus distorting the image. Instead, the “live-view capture” icon on the iPad™ screen was used. Since both of the researcher’s hands were occupied operating the microscope, an assistant aided in capturing the images by pressing the on-screen “live-view capture” icon when instructed to do so by the researcher.

Stellenbosch University https://scholar.sun.ac.za

In an attempt to standardise the conditions at which images were taken, the cultivars were investigated in the same order: Pinotage, Shiraz, Cabernet Sauvignon and Grenache noir for each imaging session. The starting time of each session was also kept similar as far as possible. In some cases, however, the order of cultivars or the general time frame of the investigations had to be altered to compensate for unexpected weather conditions, problematic ambient light conditions or the availability of an assistant. The order of the positions investigated on each leaf was always from positions 1 to 6 with three images being captured for each position before moving to the next. A graphical summary of this process is given in Figure 15.

Each image captured had a unique image number automatically assigned to it when it was saved to the iPad's™ memory. These numbers were recorded on field data sheets to be able to match each image to its corresponding leaf and observation position upon download. If the need arose for additional images to be captured for a particular position in order to compensate for poor quality images, or if the on-screen capture icon was accidentally pressed, this was also noted on the field data sheets.

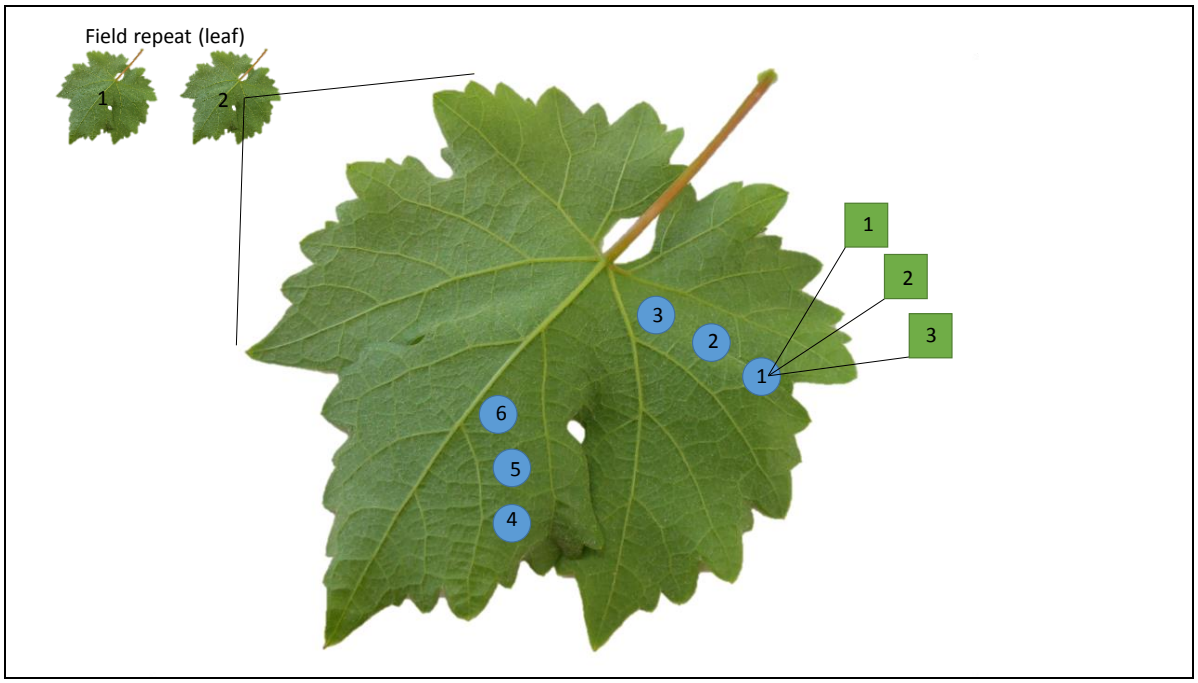

Figure 15 Summary of the levels of repetition within the study for each particular leaf position of one of the cultivars. The blue circles represent the six observation positions and the green squares represent the three images captured at each measurement position.

Imaging was done on seven different dates during the season. The General leaves used for the method comparison were imaged on 25 November 2014. The first investigation of Basal, Middle and Apical leaves took place on 3 December 2014 and this data was used to supply a larger dataset for the comparison between the image analysis methods investigated. In order to facilitate the statistical analysis of the data, not all of these sessions were included in the final dataset. The first two sessions, in which Apical 2 leaves were not investigated, were omitted and thus only five of these sessions (at roughly 13 day intervals) were included in the data analysis for the stomatal investigations, as indicated in Table 7. A timeline is also shown in Figure 16 with additional details pertaining to the leaves investigated during each session.

Stellenbosch University https://scholar.sun.ac.za

Table 7 Observation dates for the selected leaves and time lapse between them (time lapse only applicable to sessions for which the data was used in the stomatal investigations study).

Observation Session	Date	Interval between observation dates (days)
1	25/11/2014	Not applicable*
2	03/12/2014	Not applicable*
3	11/12/2014	0
4	21/12/2014	10
5	13/01/2015	23
6	22/01/2015	9
7	02/02/2015	11
Average interval length		13.25 ≈ 13

* data only used in method comparison study

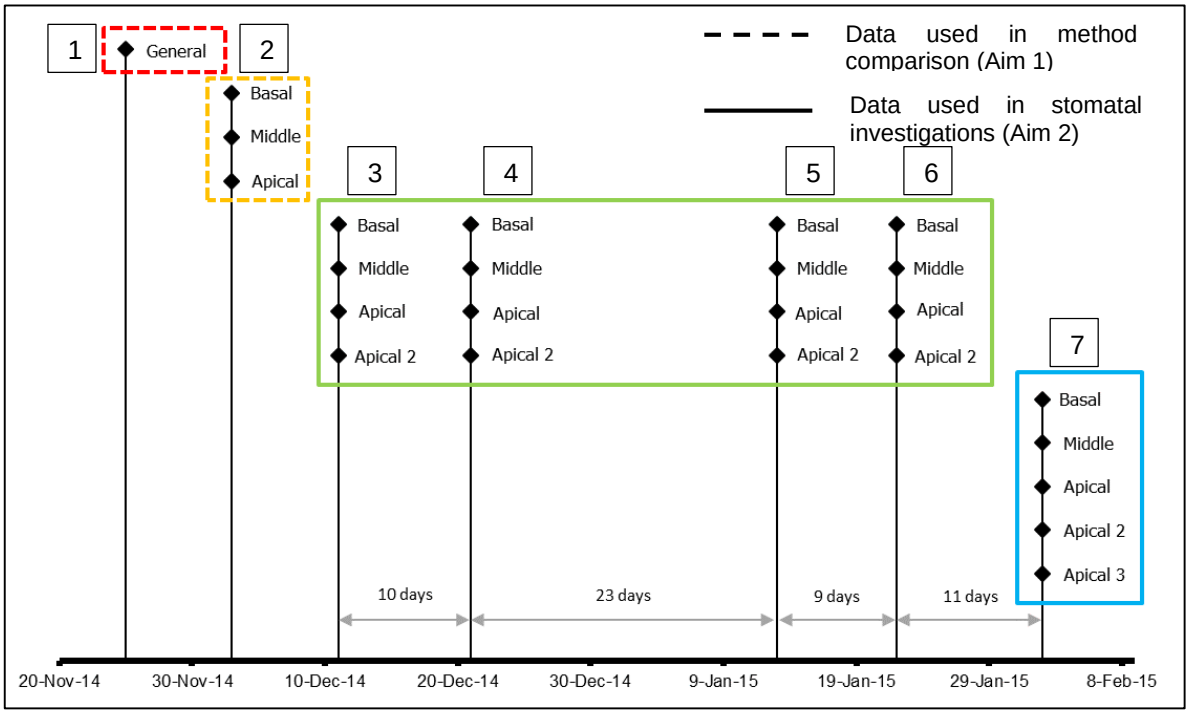

Figure 16 Timeline of all seven observation dates and investigations conducted within each session (sessions during which the same investigations were done are grouped together within the coloured blocks).

3.4 Data analysis

3.4.1 Vegetative data

The L1 length and leaf area data collected was analysed using Statistica 12 ® software (Statsoft, Tulsa, Oklahoma, USA). Graphs were drawn for each cultivar, plotting L1 length (converted to centimetre from the measured millimetre) against leaf area. By adding a regression line to such a graph the nature and strength of the relationship between the two variables can be determined and quantified. The most basic regression line represents a linear relationship between the variables on the x- and y- axes – in this case the L1 length and leaf area respectively. A strong positive linear relationship between L1 length and leaf area was

Stellenbosch University https://scholar.sun.ac.za

found to exist for all the cultivars with a goodness of fit that ranged from 89% to 94% being observed. When all the data are pooled together and a graph drawn representing the average relationship between L1 length and leaf area regardless of cultivar, the goodness of fit was 91%. An exponential regression line, however, represented the relationship even better with the goodness of fit ranging from between 94% and 97% for the individual cultivars and being close to 96% when looking at the trend in general. The correlation coefficients from which the goodness of fit was derived for the linear and exponential regressions are presented in Table 8.

Table 8 Correlation coefficients for linear and exponential relationship between L1 (main central vein) length and leaf area for the different cultivars.

Cultivar	Linear correlation coefficient (r^2)	Exponential correlation coefficient (r)
Pinotage	0.94	0.97
Shiraz	0.89	0.95
Cabernet Sauvignon	0.93	0.96
Grenache noir	0.92	0.96
All (general)	0.91	0.95

The regression lines are each represented by an equation which can be used to calculate the estimated leaf area of a particular leaf. Since the exponential regressions were better fitted to the data, their corresponding equations were selected to calculate the area of each of the leaves selected for the study. The exponential relationship graphs for each of the cultivars are shown in Figure 17 to Figure 20.

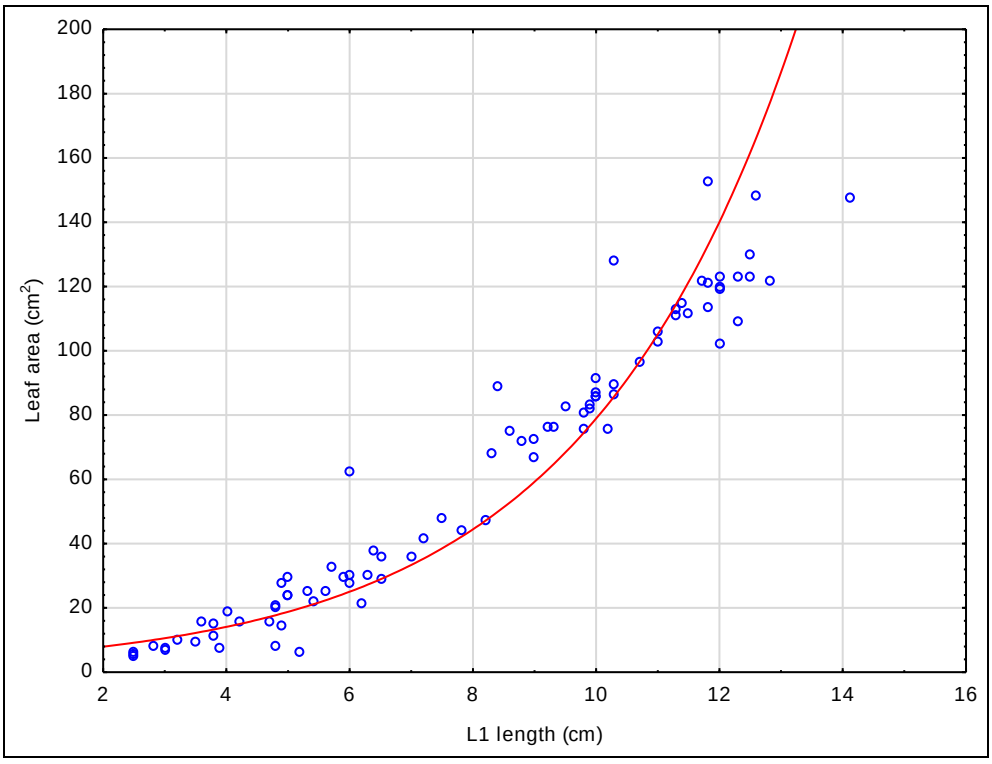

Figure 17 Exponential correlation between L1 (main central vein) length and leaf area for *Vitis vinifera* cv. Pinotage; (r=0.969 and p≤0.001) [exponential equation: Leaf area (cm^2) = 4.4756*exp(0.287*x)].

Stellenbosch University https://scholar.sun.ac.za

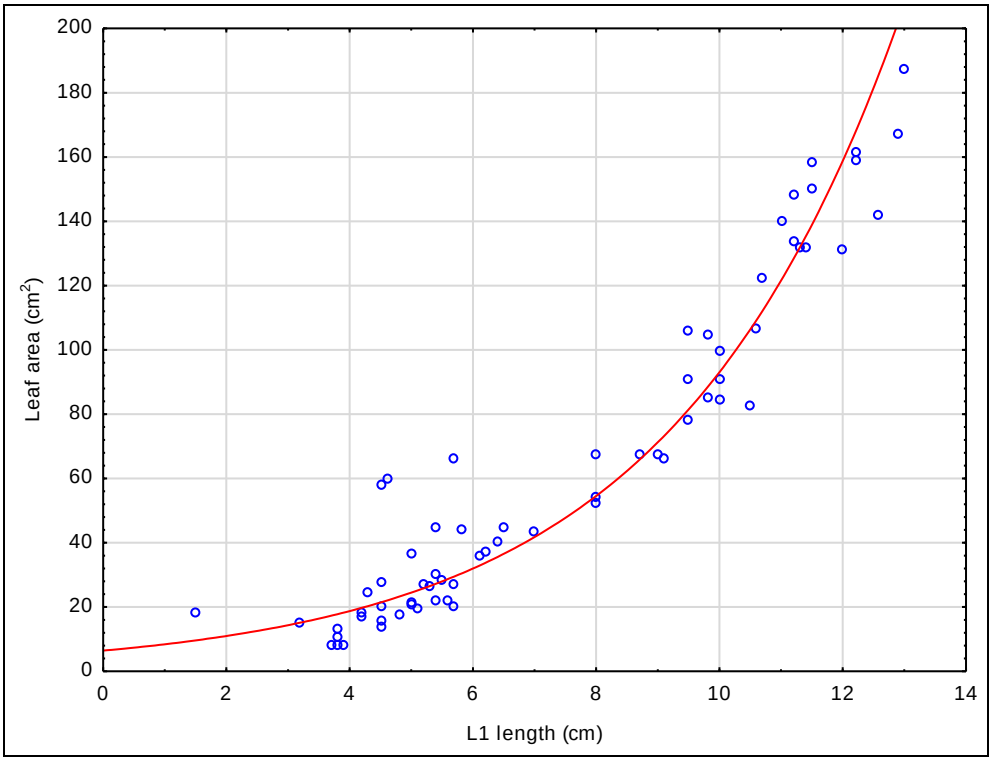

Figure 18 Exponential correlation between L1 (main central vein) length and leaf area for *Vitis vinifera* cv. Shiraz; (r=0.945 and p≤0.001) [exponential equation: Leaf area (cm^2) = 6.4402*exp(0.2671*x)].

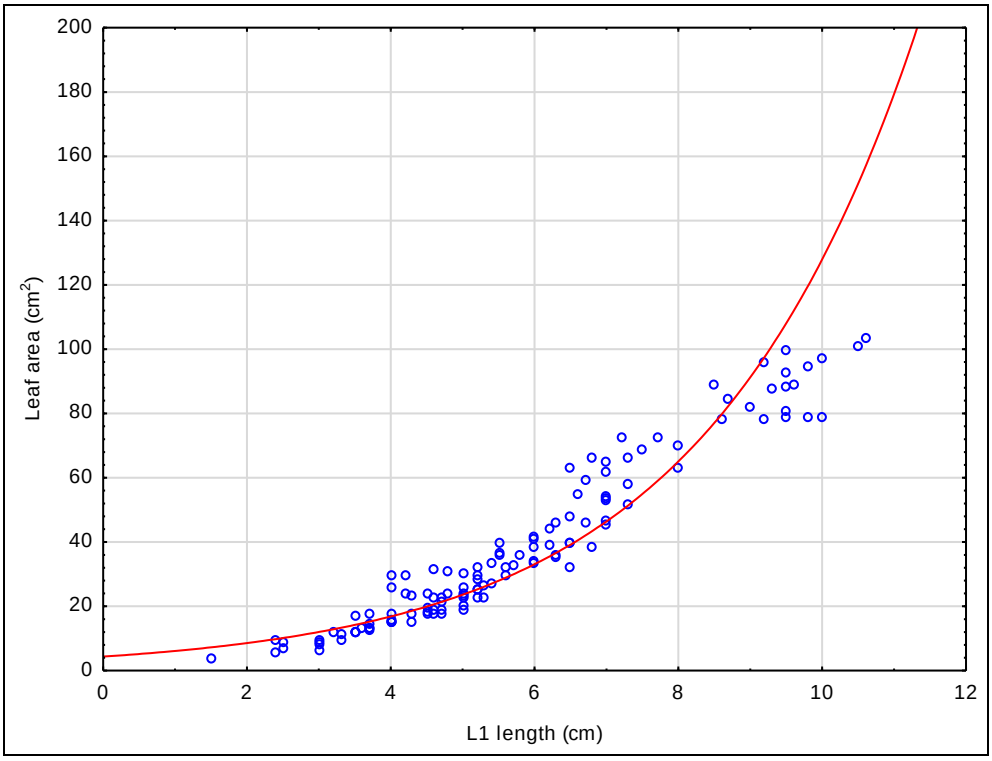

Figure 19 Exponential correlation between L1 (main central vein) length and leaf area for *Vitis vinifera* cv. Cabernet Sauvignon; (r=0.964 and p≤0.001) [exponential equation: Leaf area (cm^2) = 4.3551*exp(0.338*x)].

Stellenbosch University https://scholar.sun.ac.za

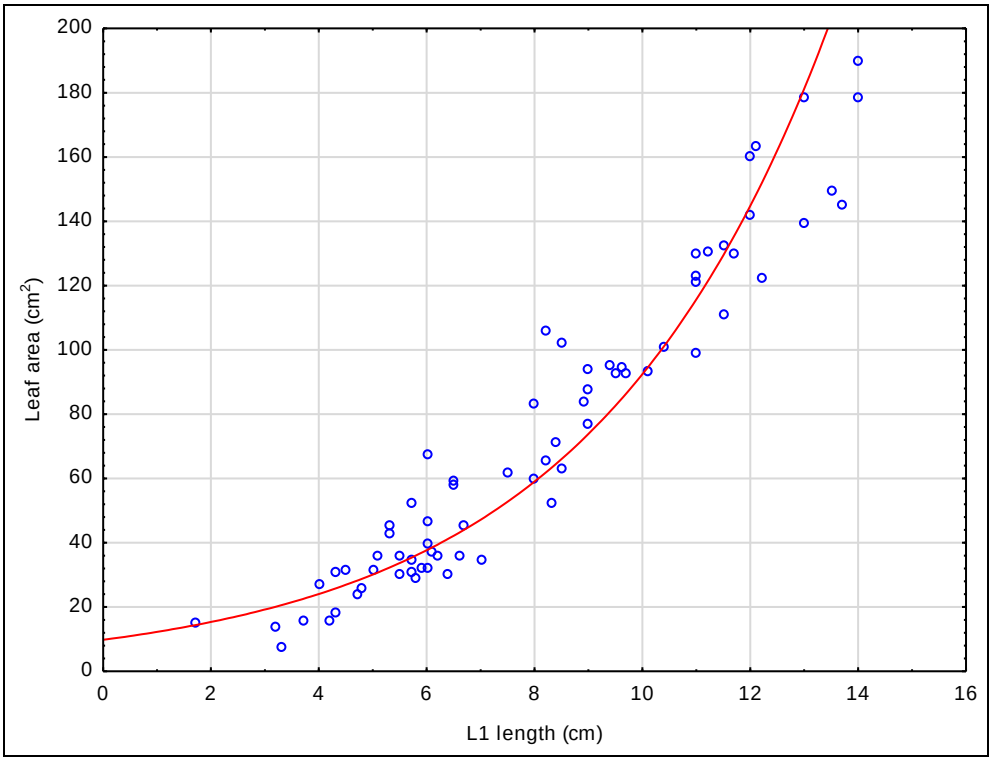

Figure 20 Exponential correlation between L1 (main central vein) length and leaf area for *Vitis vinifera* cv. Grenache noir; (r=0.958 and p≤0.001) [exponential equation: Leaf area (cm^2) = 9.8024*exp(0.2244*x)].

3.4.2 Field microscopy data

In order to count stomata, the impression method is commonly used (Weyers & Meidner, 1990). This method involves making an impression of the leaf surface with a substance like clear nail polish. A thin layer of varnish is applied to the leaf and allowed to dry. Once dry, this film is peeled off using clear adhesive tape or forceps, after which it is mounted on a slide and investigated under a microscope. This method can give valuable information regarding stomatal density and aperture, but as previously mentioned, the stomatal aperture may be affected in the process of making the impressions which can lead to faulty results (Meidner, 1981). In this study, field microscopy was used to obtain images, which were then analysed using ImageJ (Rasband, 2014) to determine stomatal density.

There was some uncertainty regarding the specific process to be followed for the image analyses. Three methods of image analyses, all very similar in principle, were tried on a smaller selection of images before deciding which would be the most suited for analysing the complete set of images. These methods will be discussed in more detail in the sections to follow and results will be shown to substantiate the choice of method with which analyses proceeded, but first we will look at the analysis process in general.

3.4.2.1 Basic principles of the image analysis process

The images were each opened in the image editing programme. Shapes, the nature of which differed between the respective methods, were drawn onto the images as a way of segmenting them into areas wherein stomata could be counted more easily. The software allows for the areas of these drawn shapes to be measured. The units in which this measurement is made can also be set by the user. In order to get the accurate area in the desired unit, a calibration image was used to quantify a line of known distance. The AirMicroPad application has a function in which a scale bar can be displayed on the image. In this case, the scale bar displayed for the

Stellenbosch University https://scholar.sun.ac.za

400x magnification used represented 100 µm. The accuracy of the application's scale was tested by checking whether it corresponded to 100 µm on a calibrated stage micrometer (Carl Zeiss Microscopy GmbH, Jena, Germany). The stage micrometer used is shown in Figure 21 and an explanation of the scale and diagrammatic representation thereof (Figure 22) follows. The slide has a 1 mm line, which is divided into 100 units of equal length, etched into the glass window – this serves as the scale.

Figure 21 Calibrated stage micrometer used to determine the accuracy of the AirMicroPad application's scale bar.

1 Division = 0.01 mm = 10 µm

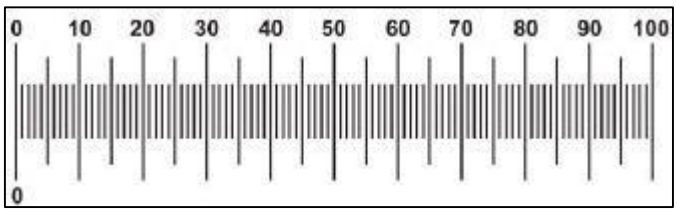

Figure 22 Detailed scale of the stage micrometer. Each minor unit has a length of 10 µm (https://www.tedpella.com/histo_html/Pro-Stage-Micrometers.html).

It is therefore expected that the 100 µm scale bar in the AirMicroPad application should correspond to the length of ten divisions (minor units). This was confirmed by viewing the scale of the stage micrometer with the microscope in such a way that it was in close proximity to the application's 100 µm scale bar on the iPad™ screen. The number of divisions corresponding to the scale bar could then be determined. An image of this scene was captured and used as a calibration image (Figure 23).

Stellenbosch University https://scholar.sun.ac.za

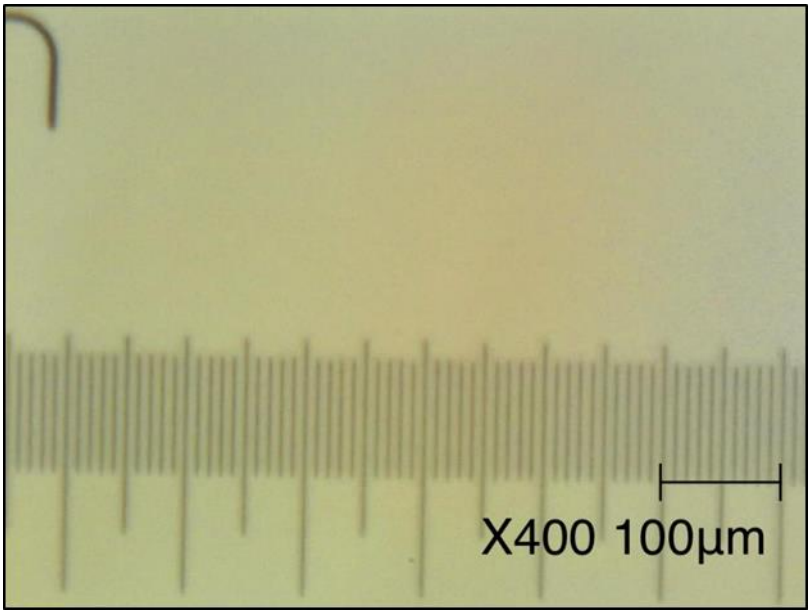

Figure 23 Calibration image showing that the 100 µm scale bar from the application corresponds to 10 divisions as expected if the scale bar is accurate.

Since the scale of the AirMicroPad application was found to be accurate, the setting of the scale in the image editing software could be done using the scale bar on the calibration image. After zooming in to enlarge the scale bar to allow for accurate measurement, its length was determined using the image editing software programme's measurement tool. The length is given in pixels and since it is known that the line represents 100 µm, the number of pixels per 100 µm can be calibrated for. It was found that this line is represented by 95.93 pixels. This value is entered into the programme's function for setting the scale (Figure 24) and all measurements that follow will then automatically be made in micrometre or square micrometre, depending on whether the length of a line or the area of a shape is measured.

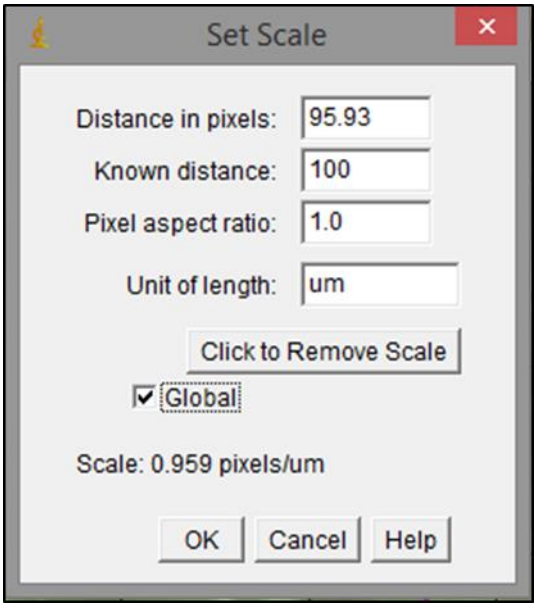

Figure 24 Screen capture showing how the scale was set in ImageJ (Rasband, 2014).

When the shapes are drawn onto the image, the measurement tool is used to measure the area of each shape and the results are displayed in a results table which can be saved as a text file. These results tables were saved per image and with that image's number in the file name, in

Stellenbosch University https://scholar.sun.ac.za

order to facilitate matching the areas with the correct shapes on the correct image. Since the common unit for expressing stomatal density is number of stomata per square millimetre (stomata/mm^2), the areas measured in square micrometre (µm^2) had to be converted to square millimetre (mm^2). To make this conversion, the measured areas were divided by a factor of 10^6.

The shapes were always drawn in the same order starting in the top left area of an image and then working in a clockwise direction. The stomata within these shapes were counted in the same order in which the shapes were drawn so as to match the stomatal counts to the correct measured area. Stomatal counts were done using the “cell counter” function of the image editing software. Provision is made in this function to count multiple sets of items by allocating specific numbered markers to related items within a particular set. A different marker was thus used to count stomata within each shape. The stomata in the first shape were counted using marker number one, those in the second shape using marker number two and so on. The stomata were identified visually by the researcher based on their unique oval shape and their white appearance (presumably due to the reflection of light). They were counted by simply clicking on or near them – the relevant numbered marker selected was then placed at this position and the click counted by the cell counter function. With every click, another identical marker is placed on the image and another count assigned to that particular marker in the cell counter function. When a different marker is selected, that numbered marker is displayed on the image when clicking and the click is now counted for the newly selected marker. Figure 25 illustrates the use of the cell counter function with an example of an image on which stomata have been counted using different markers (A), and the corresponding cell counter (B). The stomatal counts for each shape were recorded on a datasheet and the cell counter also saved should the need arise for a check to be conducted. The numbers of the markers within the shapes served another purpose – that of later matching a particular shape to its measured area in the file saved form the results table. The stomatal counts were later entered into an Excel 2013 (Microsoft Corporation, Redmond, Washington, USA) datasheet along with the areas of the shape to which they corresponded. This enabled the calculation of stomatal density by dividing the number of stomata within a shape by the area of that shape.

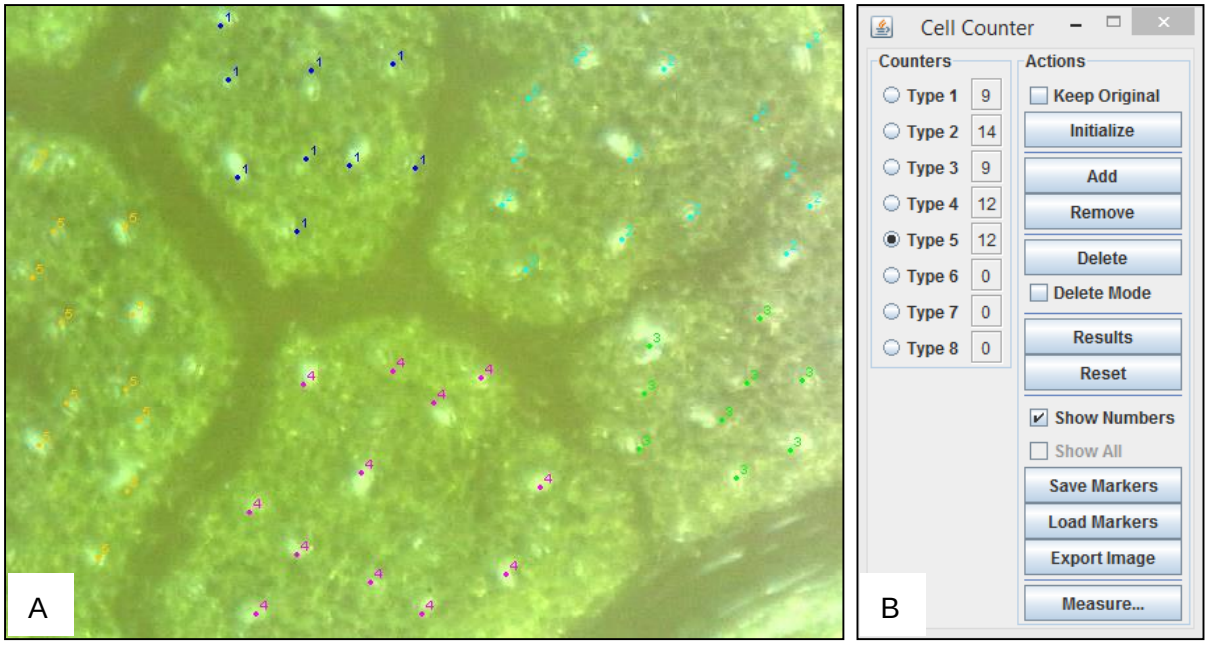

Figure 25 A) Image on which counter markers have been placed at the position of stomata B) The cell counter corresponding to the markers in A.

Stellenbosch University https://scholar.sun.ac.za

The exact process of each image analysis method will now be discussed.

3.4.2.2 Block method of analysis

It was decided that the images should be sectioned into areas in which the counting of stomata could be done more easily. In order to accomplish this, blocks were drawn onto the image in ImageJ (Rasband, 2014). The aim was to draw between four and six blocks per image. There were cases where the number of blocks drawn fell outside of this range, but this was in the minority. The blocks were drawn in such a way that they covered the largest possible area of inter-vein spaces, while still avoiding as much of the veins as possible. Problems with images could include the presence of hairs that still remained after the hair-removal process, and blurring of sections of the images. The blocks were drawn in such a manner as to avoid such problem areas as well since they would yield lower stomatal densities due to difficulty in counting stomata. Figure 26 shows an example of the use of this method to analyse an image.

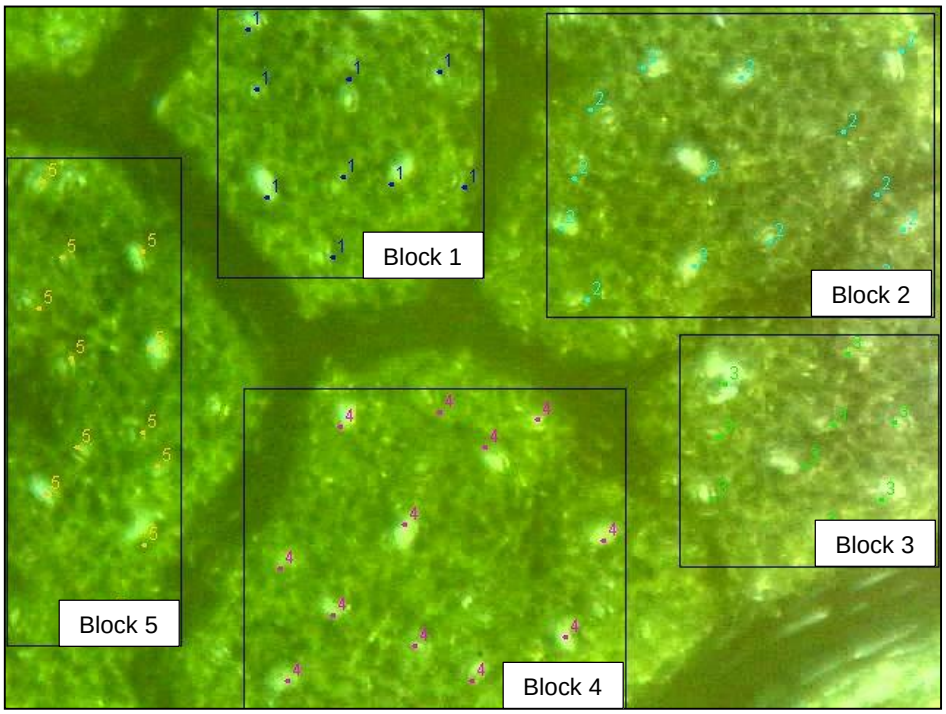

Figure 26 Example of an image analysed using the block method indicating five blocks in which stomata were counted (represented by numbered markers within the blocks).

3.4.2.3 Polygon method of analysis

Since blocks are very rigid shapes, their positioning to avoid problem areas (veins, hairs and blurred sections) often led to sections ideal for counting also not being included in the selections. Polygons, on the other hand, are far more easily manipulated since they do not have a specific shape and each side can thus vary in length and the angles between adjoining sides are not fixed.

The polygon method was used in much the same way as the block method. The shapes were drawn in the same order and the stomatal counts were also done in that order so that the measured area of each polygon could be matched to the number of stomata within that polygon. Once again the aim was to avoid veins as far as possible and also to exclude blurred sections or sections with visual obstructions. The polygons were therefore drawn within the inter-vein spaces only, totally omitting any large veins, and at the same time including the entire inter-vein

Stellenbosch University https://scholar.sun.ac.za

section that is suitable for counting. The use of the polygon method in analysing an image is shown in Figure 27.

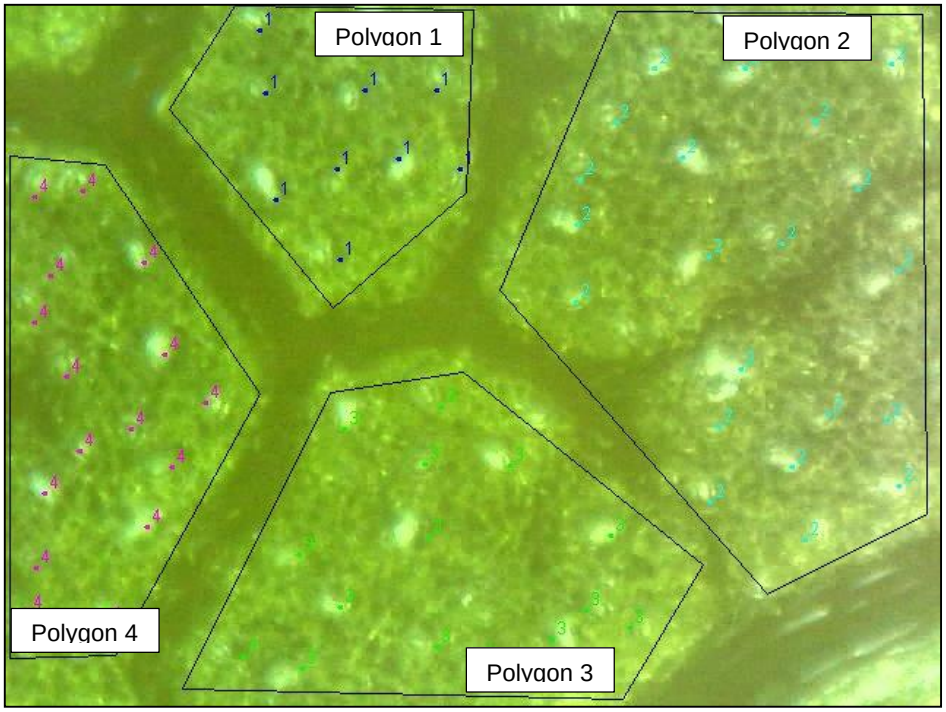

Figure 27 Example of an image analysed using the polygon method indicating four polygons in which stomata were counted (represented by numbered markers within the polygons).

3.4.2.4 Grid quadrant method of analysis

The selection of areas in which to carry out stomatal counts by the researcher, adds a level of bias and this is not ideal if accurate results are to be obtained from the analyses. The third method of analysis was developed to remove this human factor from the analysis process. In this method, the grid function available in the image editing software was used in determining over which areas counts should be conducted. The size of the grid can be set manually and a size at which the largest portion of the image was covered by whole grid blocks was chosen. At the selected size, each grid block represented 9500 μm^2. The images were then divided into four quadrants, each made up of six grid blocks (two blocks tall by three blocks across) (Figure 28). The counts were conducted in the four quadrants in the order of top left, top right, bottom left and then bottom right, and the marker numbers 1, 2, 3, and 4 assigned to these quadrants respectively. Figure 29 shows an example of an image in which the grid quadrant method was used to do stomatal counting – please note that the quadrants were not drawn in on the images as the blocks and polygons were, but that the grid squares were used as a guide instead.

Stellenbosch University https://scholar.sun.ac.za

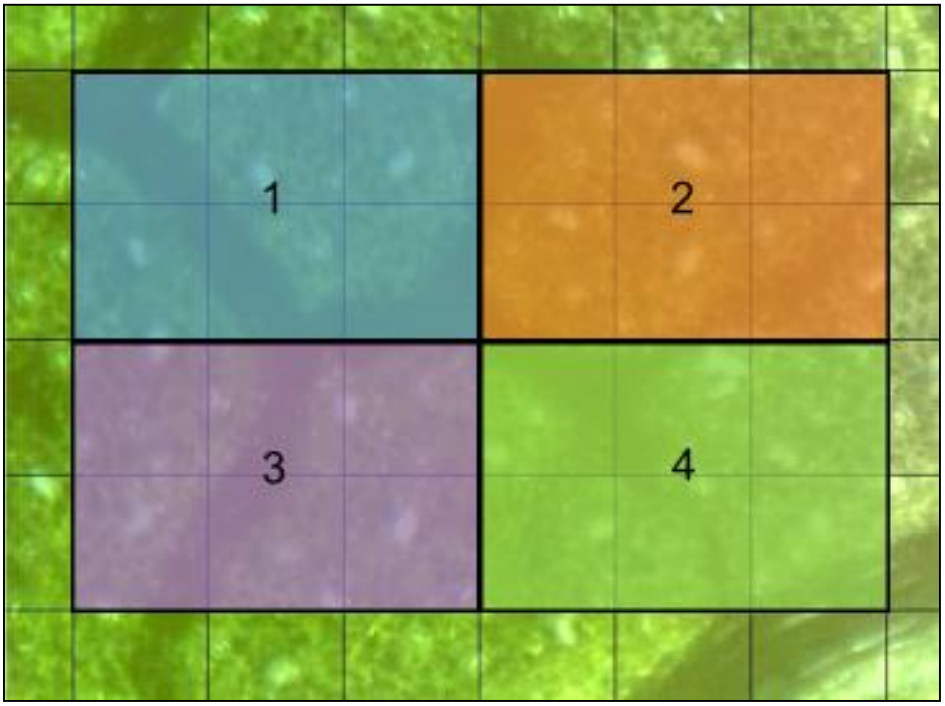

Figure 28 Image showing how the division of images into quadrants was done using the grid squares.

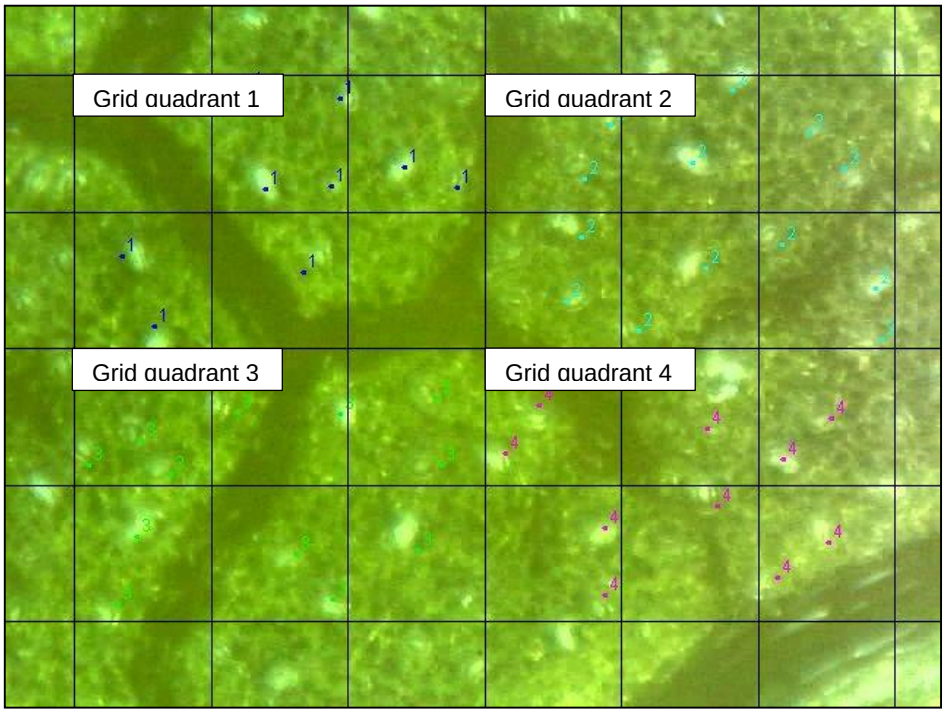

Figure 29 Example of image analysed using the grid quadrant method – note that the quadrant divisions are not drawn in (numbered markers represent the stomata counted within the quadrants).

3.5 Method comparison results

It was found that there were differences in the results obtained from the three methods of analysis. The results obtained when employing each of the three methods to analyse a single image are shown in Table 9. The average stomata/mm^2 for each method was calculated and used to compare the results. Using polygons yielded the highest average stomata/mm^2 – largely due to the fact that all veins were excluded and counts were thus conducted over inter-vein

Stellenbosch University https://scholar.sun.ac.za

spaces only. The second highest value was obtained using blocks, where the veins were also excluded to a degree, but not entirely as with the use of polygons. The lowest value was obtained using the grid quadrant method. This can be ascribed to the fact that the image was divided into standardised sections regardless of whether or not veins or visual obstructions were present in these sections. The grid quadrant method is thus unbiased and one would expect the results obtained from such an analysis method to be more representative of the actual nature of stomatal density on a leaf, than in the cases where the areas over which counts were conducted were purposefully chosen to meet some or other standard.

Table 9 Results from analysing IMG 3353 using the block, polygon and grid quadrant methods.

Image number	Analysis method	Shape Repeat	Area (µm²)	Area (mm²)	Stomatal Count	Stomatal density (stomata/mm²)	Average stomatal density (stomata/mm²)
IMG 3353	Block	1	36384.17	0.036	9	**247.36**	**249.32**
		2	59886.98	0.060	14	**233.77**	
		3	30576.91	0.031	9	**294.34**	
		4	62102.32	0.062	12	**193.23**	
		5	43183.38	0.043	12	**277.89**	
IMG 3353	Polygon	1	31831.80	0.032	9	**282.74**	**282.69**
		2	93557.11	0.094	21	**224.46**	
		3	57619.49	0.058	15	**260.33**	
		4	44048.22	0.044	16	**363.24**	
IMG 3353	Grid quadrant	1	57000.00	0.057	9	**157.90**	**192.98**
		2	57000.00	0.057	13	**228.07**	
		3	57000.00	0.057	12	**210.53**	
		4	57000.00	0.057	10	**175.44**	

The comparison between the three methods is summarised in Table 10 reporting the percentages by which the methods differed from one another.

Table 10 Comparison between the average stomata/mm² values obtained when analysing the same image (IMG 3353) using the three different methods.

Analysis method	**Average stomatal density (stomata/mm2)**	**% Difference to Block**	**% Difference to Polygon**	**% Difference to Grid quadrant**
Block	249.318		-11.805	29.192
Polygon	282.691	11.805		46.485
Grid quadrant	192.982	-29.192	-46.485	

The question was now which method gave the most realistic results and how these values compare to what has been reported in literature. This decision could not be based on the results reported above, which are based on a single image alone. We will now look at the results from the dataset obtained through the preliminary comparison for which images of the General leaves for all four cultivars from the 25 November 2014 session were analysed.

The average stomata/mm² for each method calculated over the entire dataset is shown in Figure 30. Once again the polygon method had the highest stomatal density and the grid quadrant method the lowest. The exact figures are represented in Table 11, along with the standard deviation of the stomatal density calculated for each method.

Stellenbosch University https://scholar.sun.ac.za

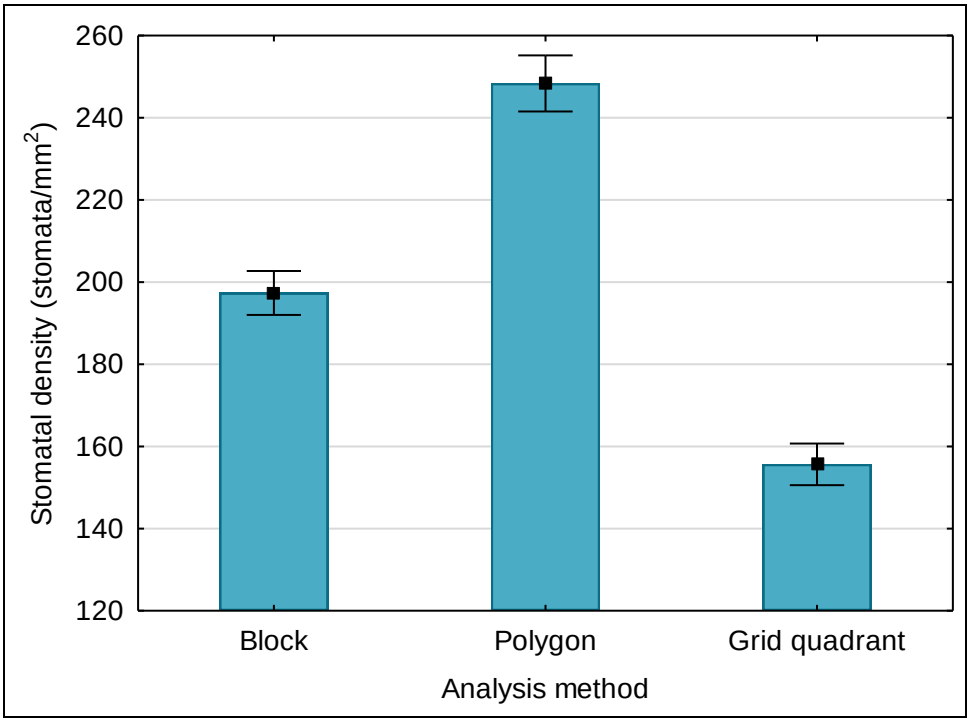

Figure 30 Mean stomatal density (stomata/mm^2) for the three image analysis methods; vertical bars denote 95% confidence intervals.

Palliotti *et al.* (2000) conducted investigations on two grapevine cultivars, namely Cabernet franc and Trebbiano Toscano. They reported stomatal densities ranging from 160.3 to 222.3 stomata/mm^2. In another study by Rogiers *et al.* (2011), the stomatal density of Chardonnay vines grown under different soil temperatures (cool and warm) and different carbon dioxide concentrations (ambient and low) were determined. For vines that were grown under ambient carbon dioxide concentration, the stomatal density was found to be between 160 and 220 stomata/mm^2. Serra *et al.* (2014) investigated Pinotage vines in a study looking at the effect which rootstocks have on drought-tolerance of grapevines. The stomatal density and stomatal aperture were two variables that were quantified. The field-grown vines were either well-watered or water constrained and leaves selected were either fully exposed to sunlight or shaded. The stomatal densities for the combinations of these conditions ranged between 96.3±6.3 and 113.8±6.3 stomata/mm^2. The results from the grid quadrant method were most comparable with the results from Serra *et al.* (2014), while the block method corresponded closely with the reported stomatal densities from Palliotti *et al.* (2000) and Rogiers *et al.* (2011). The polygon method yielded the largest values, but they are still in line with the observation of Düring (1980) that *Vitis vinifera*, in general, has a stomatal density of between 50 and 250 stomata/mm^2.

The polygon method had the largest standard deviation at 54.41. The block and grid quadrant methods had standard deviations of 41.00 and 43.66 respectively which are very similar. Thus the stomatal densities calculated for the polygon method deviated more from the mean than for the other two methods. When these deviations are looked at in context of the average stomatal densities of the associated analysis method i.e. the coefficient of variance, the grid quadrant method shows the most variation. This could be explained by the fact that the areas over which the stomatal counts were conducted were not selectively chosen, and that there is thus no control over the presence of veins. This method is the most unbiased, and although it shows greater variation than the other two methods, it may be better suited to certain investigations depending on what the required outcomes are. For instance, if the goal is to investigate

Stellenbosch University https://scholar.sun.ac.za

stomatal frequency on a whole-leaf basis, this method will be well suited, since the natural effect of veins on stomatal number is taken into account. If, however, the idea is to investigate the alteration in stomatal density in response to a certain stimulus, the block or polygon method could be employed since the changes would be notable over inter-vein areas only.

Table 11 Mean stomatal density (stomata/mm^2) and coefficient of variance thereof for the three analysis methods.

Analysis method	Average stomatal density (stomata/mm^2)	Number of shapes counted	Std. Dev.	Coefficient of variance
Block	197.36	228	41.00	20.78
Polygon	248.35	247	54.41	21.91
Grid quadrant	155.64	288	43.67	28.06

3.6 Conclusion

There are many methods of research available to investigate stomatal density, but most are destructive, relying on epidermal peels and impressions, or leaf segments which can be investigated using light microscopy or scanning electron microscopy. Field microscopy as a non-destructive alternative would allow for time related studies to be conducted and this would be very useful.

It was important to analyse the images captured during the investigations in such a way that the most realistic results, related to the purpose of the study, could be obtained. In order to decide which of the three image analysis methods would be the best to use, a comparison had to be made between the results obtained from each method. A preliminary comparison was made using images from the session conducted on 25 November 2014. A further comparison was then conducted using the images for the different leaf position leaves from the 3 December 2014 session as well. These images were manually checked to determine which were of the best quality - the selected images would typically not have blurred areas or hair (or other obstructions to a clear view of the leaf surface) present. From these selected images a further selection was made to represent comparable sets of investigations as far as possible – the aim was to be able to use images from the same leaf repeat number for all leaf positions as well as from the same observation positions. There were, however, some cases where a different leaf repeat or observation position's images had to be used.

To facilitate the counting of stomata, it would be beneficial to divide each image into smaller areas in which to conduct the counting. It is very easy to become biased during this process. The block method had some degree of bias due to the fact that the veins were excluded as far as possible. The polygon method was by far the most biased since no portions of veins were included in the selected areas. The polygon method's stomatal density results were also the highest. Since the grid quadrant method made use of a grid function in which standardized areas were selected for stomatal counts, one would expect this method to give the most representative stomatal densities with regard to the whole leaf surface. This method did, however, have the greatest level of variation, but this is to be expected since the areas for counting are not manipulated in any way. As previously mentioned, the method of analysis should be chosen with the purpose of the investigation in mind. In this study, the aim was to determine stomatal density as a whole, and thus it would be more accurate if the natural effect

Stellenbosch University https://scholar.sun.ac.za

of veins on stomatal density is taken into account. Furthermore, there is large variation between grapevine cultivars with regard to their leaf characteristics – particularly leaf size, vein length, the ratio between main and lateral veins, as well as the angles between veins (Bodor *et al.*, 2012). These characteristics may affect stomatal density by the distribution and amount of veins, further supporting the use of the grid quadrant method. This was the method which was chosen for conducting stomatal counts in this study.

3.7 Literature cited

Bodor, P., Baranyai, L., Balo, B., Toth, E., Strever, A.E., Hunter, J.J. & Bisztray, G.D., 2012. GRA.LE.D (GRApevine LEaf Digitalization) Software for the detection and graphic reconstruction of ampelometric differences between *Vitis* leaves. S. Afr. J. Enol. Vitic. 33(1), 1–6.

Düring, H., 1980. Stomatafrequenz bei Blättern von *Vitis*-Arten und -Sorten. Vitis 19, 91–98.

Doheny-Adams, T., Hunt, L., Franks, P.J., Beerling, D.J. & Gray, J.E., 2012. Genetic manipulation of stomatal density influences stomatal size, plant growth and tolerance to restricted water supply across a growth carbon dioxide gradient. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 367(1588), 547–555.

Gerzon, E., Biton, I., Yaniv, Y., Zemach, H., Netzer, Y., Schwartz, A., Fait, A. & Ben-Ari, G., 2015. Grapevine anatomy as a possible determinant of isohydric or anisohydric behavior. Am. J. Enol. Vitic. 66(3), 340–347.

Meidner, H., 1981. Measurements of stomatal aperture and responses to stimuli. In: Jarvis, P.G. & Mansfield, T.A. (eds). Stomatal physiology. Cambridge University Press, Cambridge. pp. 25–49.

Nothnagle, P.E., Chambers, W., Fellers, T.J. & Davidson, M.W., 2015. Reflected (episcopic) light illumination. Available from: https://www.microscopyu.com/articles/stereomicroscopy/stereoreflected.html [accessed 25 Jul 2015].

Palliotti, A., Cartechini, A. & Ferranti, F., 2000. Morpho-anatomical and physiological characteristics of primary and lateral shoot leaves of Cabernet Franc and Trebbiano Toscano grapevines under two irradiance regimes. Am. J. Enol. Vitic. 51(2), 122–130.

Pratt, C., 1974. Vegetative anatomy of cultivated grapes - a review. Am. J. Enol. Vitic. 25(3), 131–150.

Rasband, W.S., 2014. ImageJ. U.S. National Institutes for Health, Bethesda, Maryland, USA. Available from: http://imagej.nih.gov/ij/

Rogiers, S.Y., Hardie, W.J. & Smith, J.P., 2011. Stomatal density of grapevine leaves (*Vitis vinifera* L.) responds to soil temperature and atmospheric carbon dioxide. Aust. J. Grape Wine Res. 17(2), 147–152.

Sade, N., Gebremedhin, A. & Moshelion, M., 2012. Risk-taking plants: anisohydric behavior as a stress-resistance trait. Plant Signal. Behav. 7(7), 767–770.

Schultz, H.R., 2003. Differences in hydraulic architecture account for near-isohydric and anisohydric behaviour of two field-grown *Vitis vinifera* L. cultivars during drought. Plant Cell Environ. 26(8), 1393–1405.

Serra, I., Strever, A., Myburgh, P.A. & Deloire, A., 2014. Review: the interaction between rootstocks and cultivars (*Vitis vinifera* L.) to enhance drought tolerance in grapevine. Aust. J. Grape Wine Res. 20(1), 1–14.

Stellenbosch University https://scholar.sun.ac.za

Soar, C.J., Speirs, J., Maffei, S.M., Penrose, A.B., McCarthy, M.G. & Loveys, B.R., 2006. Grape vine varieties Shiraz and Grenache differ in their stomatal response to VPD: apparent links with ABA physiology and gene expression in leaf tissue. Aust. J. Grape Wine Res. 12(1), 2–12.

Tramontini, S., Düring, J., Vitali, M., Ferrandino, A., Stoll, M. & Lovisolo, C., 2014. Soil water-holding capacity mediates hydraulic and hormonal signals of near-isohydric and near-anisohydric *Vitis* cultivars in potted grapevines. Funct. Plant Biol. 41(11), 1119–1128.

Weyers, J.D.B. & Meidner, H., 1990. Methods in Stomatal Research. Longman Scientific & Technical, England.

Stellenbosch University https://scholar.sun.ac.za

Chapter 4

Research results

Stomatal density and stomatal number per leaf investigated in four cultivars of *Vitis vinifera* L.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER IV: STOMATAL DENSITY AND STOMATAL NUMBER PER LEAF INVESTIGATED IN FOUR CULTIVARS OF *VITIS VINIFERA* L.

4.1 Introduction

There are many factors that have been found to influence stomatal density and among the factors often investigated are ambient CO_2 concentration and temperature. Studies concerned with these effects are often conducted on the model plant *Arabidopsis thaliana,* allowing for a molecular aspect to be added. Thus the process of stomatal development is actually investigated quite closely. It has become clear through such studies that stomatal development, and therefore also stomatal density, is greatly controlled through gene responses and interactions (Pillitteri & Torii, 2012). The environmental influences are perceived on the gene level after which responses occur bringing about the change in stomatal density. Some of the environmental factors which have been studied include light quantity and quality, CO_2 concentration, humidity and temperature (Assmann, 1988; Casson & Gray, 2008; Casson & Hetherington, 2010; Arve *et al.*, 2011). In addition to stomatal development, these external stimuli may also affect stomatal functioning by inducing stomatal closing or opening. Since stomata are the gateway for gaseous exchange, their role in drought resistance and water use efficiency has also enjoyed much attention. In the study by Xu & Zhou (2008) it was found that both stomatal density and size are affected by water deficit. A short-term response to water scarcity is the closing of stomata and this has been found to be brought about by, amongst other mechanisms, the production of abscisic acid (ABA) (Assmann & Shimazaki, 1999; Arve *et al.*, 2011). Wang *et al.* (2007) noted that stomata are crucial in optimising plant water use efficiency.

Few studies have been conducted on stomatal density of *Vitis vinifera* specifically, but Rogiers *et al.* (2011) have done some work investigating the effect of CO_2 concentration and soil temperature. Palliotti *et al.* (2000) also conducted studies on grapevines, where the effect of shade on main and lateral leaves of two cultivars was investigated. The methods that were used in these studies were destructive, meaning that a particular measurement or investigation can only be conducted once on each sample.

This study investigates the use of field microscopy as a non-destructive alternative to conventional destructive methods. This method will allow for studies to be conducted on the same samples (leaves) over a period of time. It will thus be possible to determine whether the stomatal density and stomatal number per leaf of a particular leaf changes over time – something which has not yet been investigated. Furthermore the study will aim to answer the questions of whether stomatal density differs between cultivars, between leaves of the same plant and at different chosen investigation positions on a leaf.

Please refer to Chapter 3 for full details on the study layout, methods and analysis procedure followed.

Stellenbosch University https://scholar.sun.ac.za

4.2 Statistical design and analysis

4.2.1 Statistical design

In this study there were four factors investigated, each at various levels. Table 12 represents the factors and their levels for the study period from which results were analysed (refer to Chapter 3). Based on the factors and their levels, the study can be described with a four-way factorial design (five dates, four cultivars, five leaf positions and six observation positions).

Table 12 Factors investigated and corresponding levels in the study.

	FACTORS			
	Date	Cultivar	Leaf position	Observation position
LEVELS	11/12/2014	Pinotage	Basal	1
	21/12/2014	Shiraz	Middle	2
	13/01/2015	Cabernet Sauvignon	Apical	3
	22/01/2015	Grenache noir	Apical 2	4
	02/02/2015		Apical 3	5
				6

In a four-way factorial design, there is the possibility of the four main factors independently having an effect on the dependent variable, as well as that numerous interactions may bring about an effect. Table 13 lists all the possible interactions which can be present in this study. There is the possibility of having two factors, three factors or four factors interacting with one another.

Table 13 Various possible interactions between the four factors (Date, Cultivar, Leaf position and Observation position).

	Factors interacting
2-Factor interactions	Cultivar x Leaf position
	Cultivar x Observation position
	Leaf position x Observation position
	Cultivar x Date
	Leaf position x Date
	Observation position x Date
3-Factor interactions	Cultivar x Leaf position x Observation position
	Cultivar x Leaf position x Date
	Cultivar x Observation position x Date
	Leaf position x Observation position x Date
4-Factor interaction	Cultivar x Leaf position x Observation position x Date

Stellenbosch University https://scholar.sun.ac.za

4.2.2 Statistical analysis

Statistica 12 ® (Statsoft, Tulsa, Oklahoma, USA) was used to perform a four-way analysis of variance (ANOVA). The four-factor interaction was found not to affect stomatal density or stomatal number per leaf significantly (p=0.961 and p=0.998 respectively) and it was thus omitted in order to speed up further analysis. Following the ANOVA's, a Fischer's least significant difference (LSD) post-hoc test was performed.

Apical 3 leaves were only investigated on the final day of observation and thus the data from this date was analysed separately. A prerequisite of an ANOVA is that the sample sizes must be the same (Ireland, 2010). Since the Apical 3 leaves did not have the same number of observations as the other leaf positions, it could only be analysed in conjunction with these other leaves for the single date on which it was measured.

4.3 Results

4.3.1 ANOVA results from mixed effects tests

4.3.1.1 Stomatal density as dependent variable

The ANOVA tables obtained from the fixed effect tests for the stomatal density data are presented in Table 14 and Table 15. Table 14 was produced by analysing data from all observation dates for Basal, Middle, Apical and Apical leaf positions. Apical 3 leaves were only added on the last observation date and thus Table 15 was produced from data of this last date only.

Table 14 ANOVA table obtained from analysing stomatal density data of Basal, Middle, Apical and Apical 2 leaf positions for all observation dates.

	Num DF[1]	Den DF[2]	F	p
Main Effect				
Cultivar	3	4	11.411	0.020 *[3]
Leaf position	3	12	18.081	0.000 ***
Observation Position	5	20	7.103	0.001 ***
Date	4	16	12.061	0.000 ***
Interactive Effect				
Cultivar x Leaf position	9	12	0.323	0.951 ns
Cultivar x Observation Position	15	20	0.465	0.933 ns
Leaf position x Observation Position	15	607	2.573	0.001 ***
Cultivar x Date	12	16	1.446	0.242 ns
Leaf position x Date	12	607	2.703	0.001 ***
Observation Position x Date	20	607	0.916	0.567 ns
Cultivar x Leaf position x Observation Position	45	607	1.959	0.000 ***
Cultivar x Leaf position x Date	36	607	1.521	0.028 *
Cultivar x Observation Position x Date	60	607	0.746	0.922 ns
Leaf position x Observation Position x Date	60	607	0.807	0.849 ns

[1]Degrees of freedom of the numerator.

[2]Degrees of freedom of the denominator.

[3]ns, *, ** and *** indicate not significant and significant at the 0.05, 0.01 and 0.001 levels of probability respectively.

Stellenbosch University https://scholar.sun.ac.za

Table 15 ANOVA table obtained from analysing stomatal density data of Basal, Middle, Apical, Apical 2 and Apical 3 leaf positions for the final observation date only.

	Num DF[1]	Den DF[2]	F	p
Main Effect				
Cultivar	3	4	3.976	0.108 [ns3]
Leaf position	4	16	32.470	0.000 ***
Observation Position	5	20	2.395	0.074 [ns]
Interactive Effect				
Cultivar x Leaf position	12	16	2.859	0.026 *
Cultivar x Observation Position	15	20	0.914	0.564 [ns]
Leaf position x Observation Position	20	80	2.058	0.013 *
Cultivar x Leaf position x Observation Position	60	80	1.017	0.468 [ns]

[1]Degrees of freedom of the numerator.
[2]Degrees of freedom of the denominator.
[3]ns, *, ** and *** indicate not significant and significant at the 0.05, 0.01 and 0.001 levels of probability respectively.

4.3.1.2 Estimated stomatal number per leaf as dependent variable

Table 16 and Table 17 represent the ANOVA tables obtained from analysing stomatal number per leaf data. Table 16 was produced by analysing data from all observation dates for Basal, Middle, Apical and Apical 2 leaf positions. Apical 3 leaves were only added on the last observation date and thus Table 17 was produced from data of this last date only.

Table 16 ANOVA table obtained from analysing stomatal number per leaf data of Basal, Middle, Apical and Apical 2 leaf positions for all observation dates.

	Num DF[1]	Den DF[2]	F	p
Main Effect				
Cultivar	3	4	5.221	0.072 [ns3]
Leaf position	3	12	16.922	0.000 ***
Observation Position	5	20	5.331	0.003 **
Date	4	16	3.426	0.033 *
Interaction Effect				
Cultivar x Leaf position	9	12	3.833	0.017 *
Cultivar x Observation Position	15	20	0.503	0.910 [ns]
Leaf position x Observation Position	15	607	1.024	0.427 [ns]
Cultivar x Date	12	16	3.045	0.020 *
Leaf position x Date	12	607	12.148	0.000 ***
Observation Position x Date	20	607	0.719	0.809 [ns]
Cultivar x Leaf position x Observation Position	45	607	1.536	0.016 *
Cultivar x Leaf position x Date	36	607	2.798	0.000 ***
Cultivar x Observation Position x Date	60	607	0.780	0.885 [ns]
Leaf position x Observation Position x Date	60	607	0.728	0.938 [ns]

[1]Degrees of freedom of the numerator.
[2]Degrees of freedom of the denominator.
[3]ns, *, ** and *** indicate not significant and significant at the 0.05, 0.01 and 0.001 levels of probability respectively.

Stellenbosch University https://scholar.sun.ac.za

Table 17 ANOVA table obtained from analysing stomatal number per leaf data of Basal, Middle, Apical, Apical 2 and Apical 3 leaf positions for the final observation date only.

	Num DF[1]	Den DF[2]	F	p
Main Effect				
Cultivar	3	4	3.470	0.130 [ns3]
Leaf position	4	16	20.120	0.000 ***
Observation Position	5	20	1.491	0.237 ns
Interaction Effect				
Cultivar x Leaf position	12	16	3.231	0.015 *
Cultivar x Observation Position	15	20	0.571	0.864 ns
Leaf position x Observation Position	20	80	1.147	0.322 ns
Cultivar x Leaf position x Observation Position	60	80	0.504	0.997 ns

[1]Degrees of freedom of the numerator.
[2]Degrees of freedom of the denominator.
[3]ns, *, ** and *** indicate not significant and significant at the 0.05, 0.01 and 0.001 levels of probability respectively.

4.3.2 Observations over time

There was a significant main effect between different dates with regard to stomatal density, with the first two dates having values that were higher than those of the other three dates. There was also variation in stomatal number per leaf over time, but only that of the second observation date differed significantly. Figure 31 and Figure 32 below represent these findings.

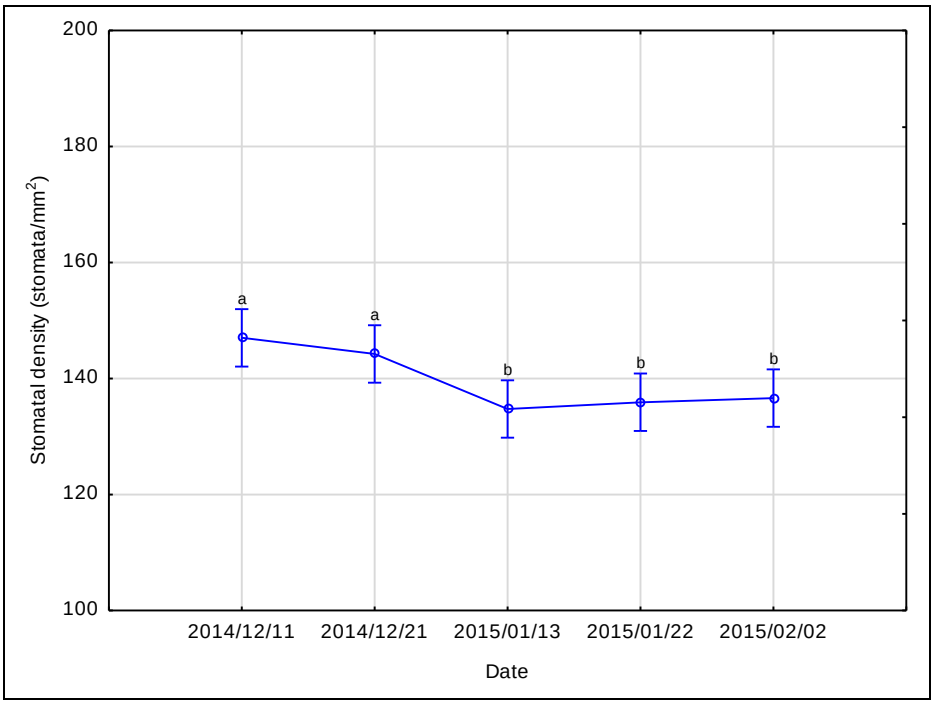

Figure 31 Mean stomatal density over time during the study period calculated by pooling data from all leaf positions of all cultivars for each observation date (p≤0.001); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

Stellenbosch University https://scholar.sun.ac.za

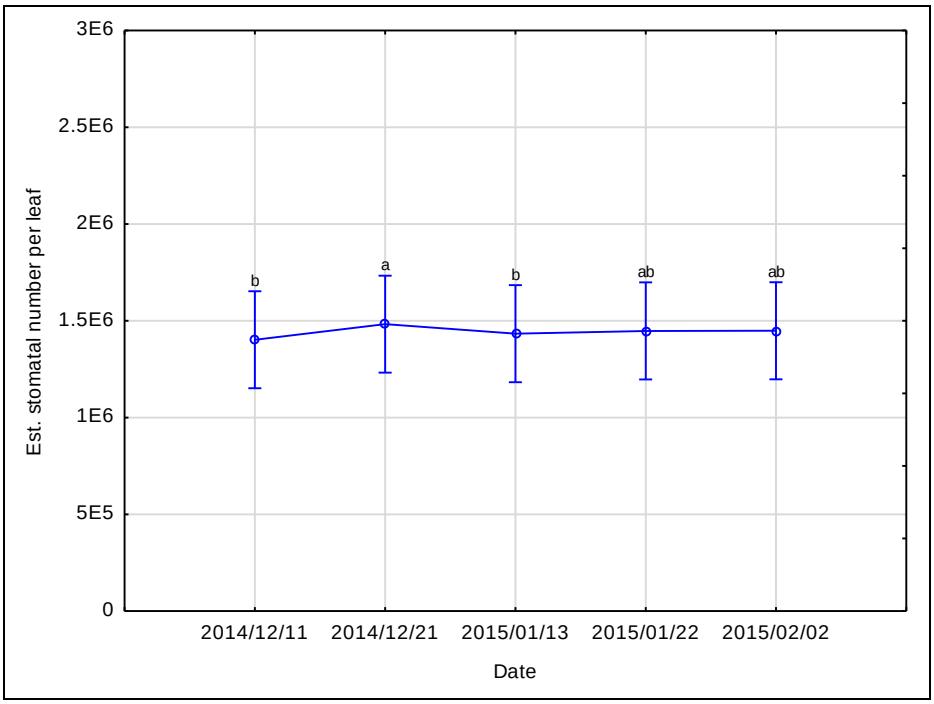

Figure 32 Mean estimated stomatal number per leaf over time for the study period calculated by pooling data from all observation positions of all leaf positions of all cultivars for each observation date ($p \leq 0.050$); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

4.3.3 Observations between cultivars

Some cultivars were found to have an effect on both stomatal density and stomatal number per leaf, but the main effect was only significant for stomatal density. The stomatal density of Cabernet Sauvignon, Shiraz and Pinotage were similar, it tended to be a little higher for Pinotage (although not significantly). Grenache noir had the highest stomatal density (Figure 33). Stomatal number per leaf for Pinotage and Grenache noir were similar and Shiraz and Cabernet Sauvignon exhibited the highest and lowest values respectively (Figure 34).

Stellenbosch University https://scholar.sun.ac.za

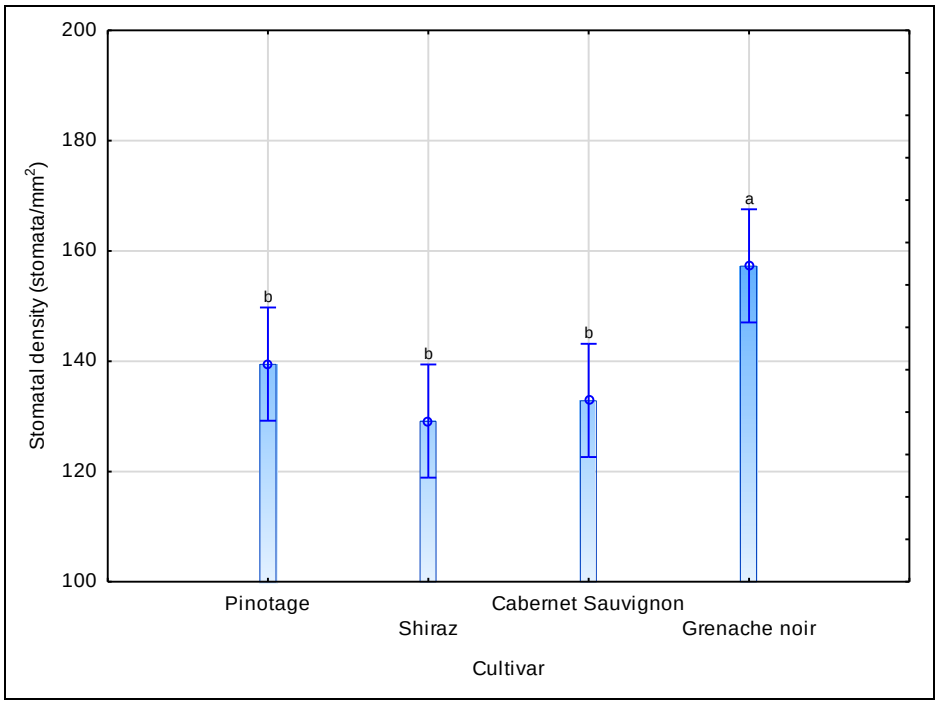

Figure 33 Mean stomatal density for the different cultivars in the study calculated by pooling data from all observation positions and all leaf positions for each cultivar ($p \leq 0.05$); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

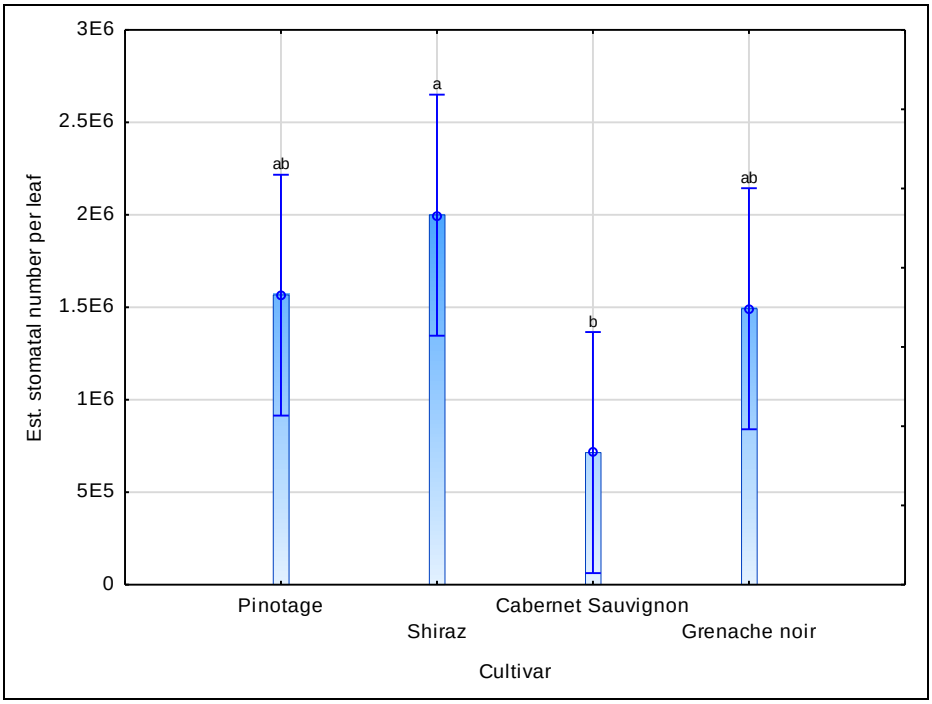

Figure 34 Mean estimated stomatal number per leaf for the different cultivars in the study calculated by pooling data from all observation positions of all leaf positions for each cultivar ($p \geq 0.05$); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

4.3.4 Observations between different leaf positions

Figure 35 and Figure 36 show the effect of leaf position on stomatal density and stomatal number per leaf respectively. The main effect of leaf position was significant for both variables. Basal, Middle and Apical leaves had a similar stomatal density and it increased significantly for Apical 2 and Apical 3 leaves. Stomatal number per leaf was found to decrease as the leaf

Stellenbosch University https://scholar.sun.ac.za

position moved further away from the base of the shoot (younger leaves). Apical and Apical 2 leaves had a similar number of stomata per leaf.

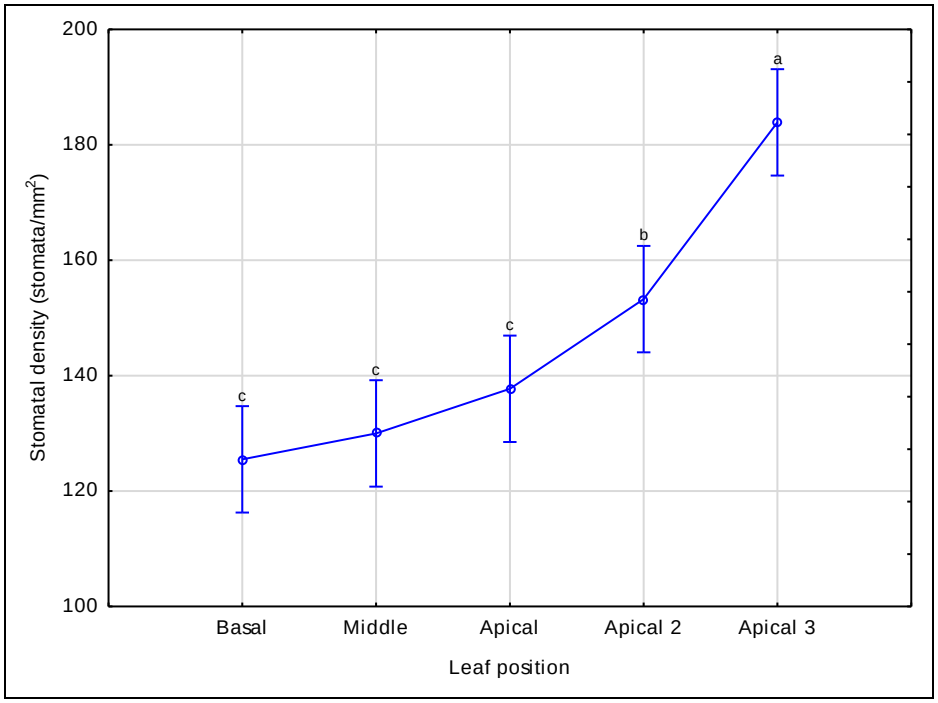

Figure 35 Mean stomatal density of the different leaf positions calculated by pooling data from all cultivars and observation positions for each leaf position (last observation date only) [p≤0.001]; vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

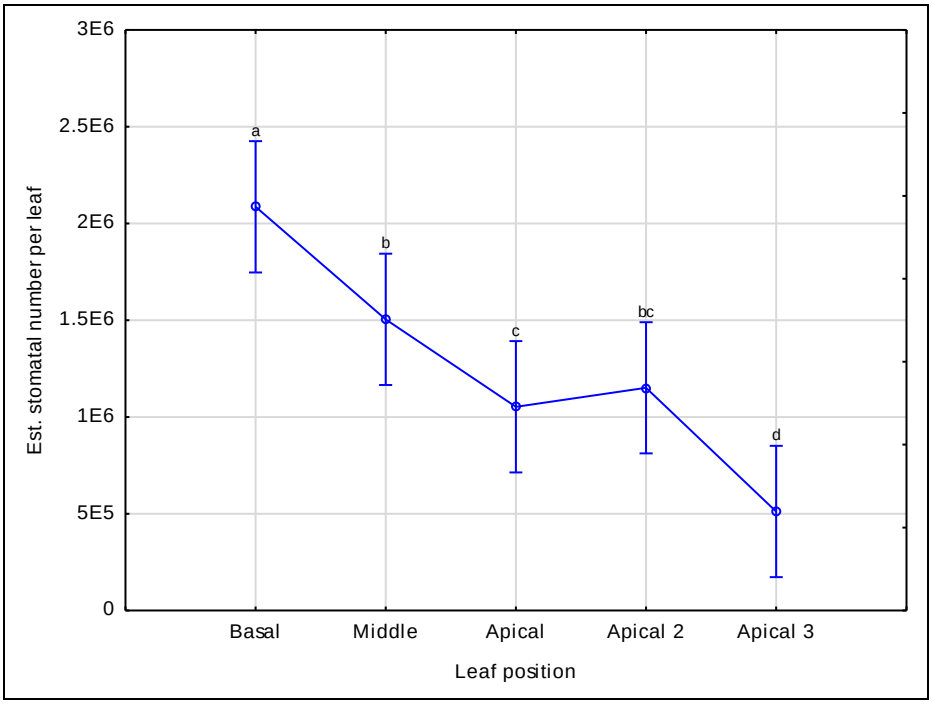

Figure 36 Mean estimated stomatal number per leaf of the different leaf positions calculated by pooling data from all cultivars and observation positions for each leaf position (last observation date only) [p≤0.001]; vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

It should be noted that Figure 35 and Figure 36 represented data from the last date only, since Apical 3 leaves were not included in the study prior to this date. Although it was a much smaller

Stellenbosch University https://scholar.sun.ac.za

data set, the stomatal density and stomatal number per leaf values of the Basal, Middle, Apical and Apical 2 leaves are comparable with those from the dataset including all investigation dates. These results are shown in Figure 37 and Figure 38 below. The only difference is that for the larger dataset, the stomatal density of the Apical leaves differed significantly from that of the Basal leaves (Figure 37). The main effect of leaf position was again significant for both variables.

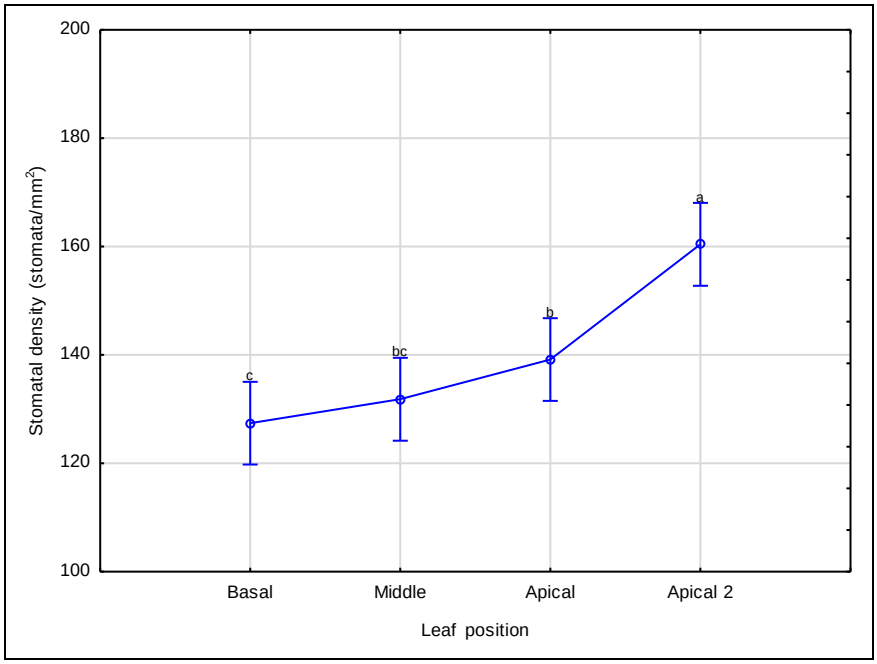

Figure 37 Mean stomatal density of the different leaf positions calculated by pooling data from all observation dates, cultivars and observation positions for the presented leaf positions (p≤0.001); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

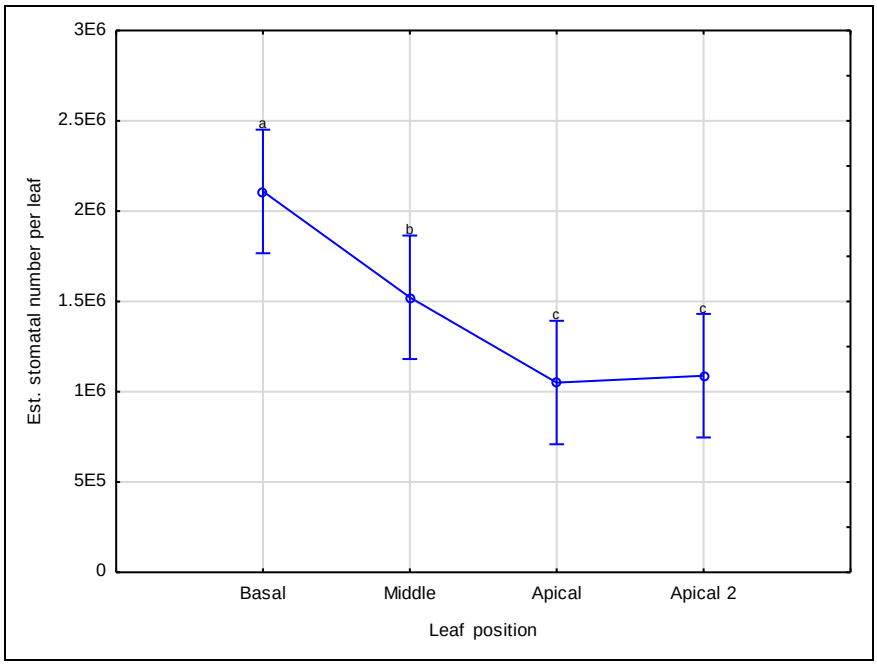

Figure 38 Mean estimated stomatal number per leaf of the different leaf positions calculated by pooling data from all observation dates, cultivars and obsevation positions for the presented leaf positions (p≤0.001); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

Stellenbosch University https://scholar.sun.ac.za

4.3.5 Observations of various factors in combination

4.3.5.1 Cultivar x Date

The interaction effect of cultivar and date on stomatal density was found to be not significant (Figure 39). In Figure 31 the values of the first two dates were higher from the rest of the dates – this is not seen in Figure 39, except for Grenache noir having a higher stomatal density on the first date. This graph does however confirm the consistently higher stomatal density of Grenache noir over the entire period as seen in Figure 33.

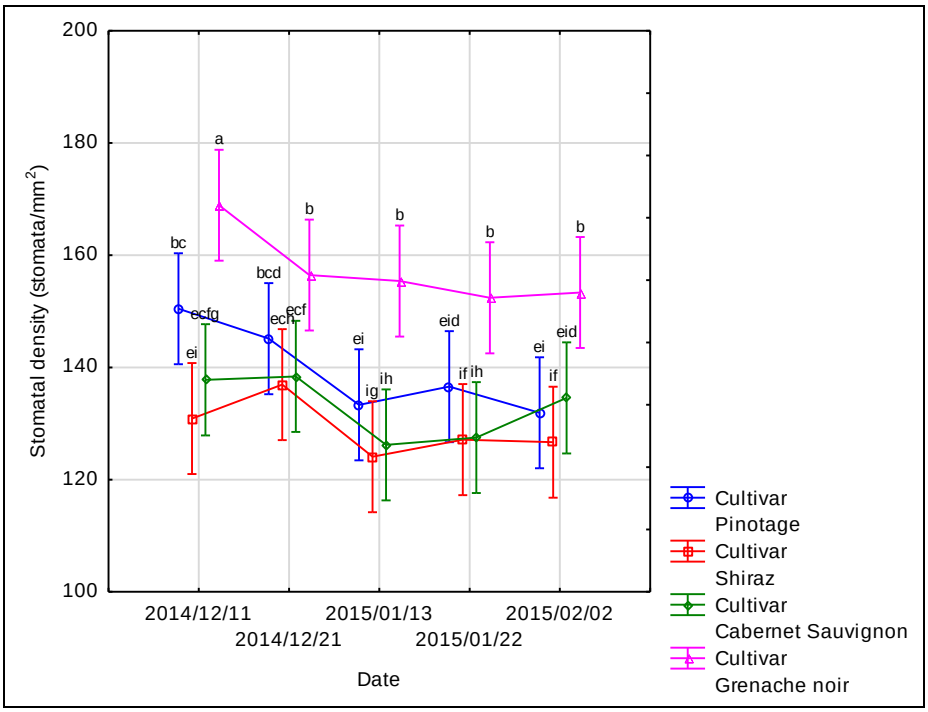

Figure 39 Mean stomatal density of the different cultivars over time for the study period calculated by pooling data from all observation positions and leaf positoins for each cultivar ($p \geq 0.050$); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

The results of the interactive effect of cultivar and date on stomatal number per leaf are shown in Figure 40, where a significant interaction effect was visible. There were differences between some of the cultivars and also between some dates, but once again only for certain cultivars. Shiraz and Cabernet Sauvignon had values significantly different from each other for the entire investigative period. For these two cultivars the stomatal number per leaf was lower on the first date.

Stellenbosch University https://scholar.sun.ac.za

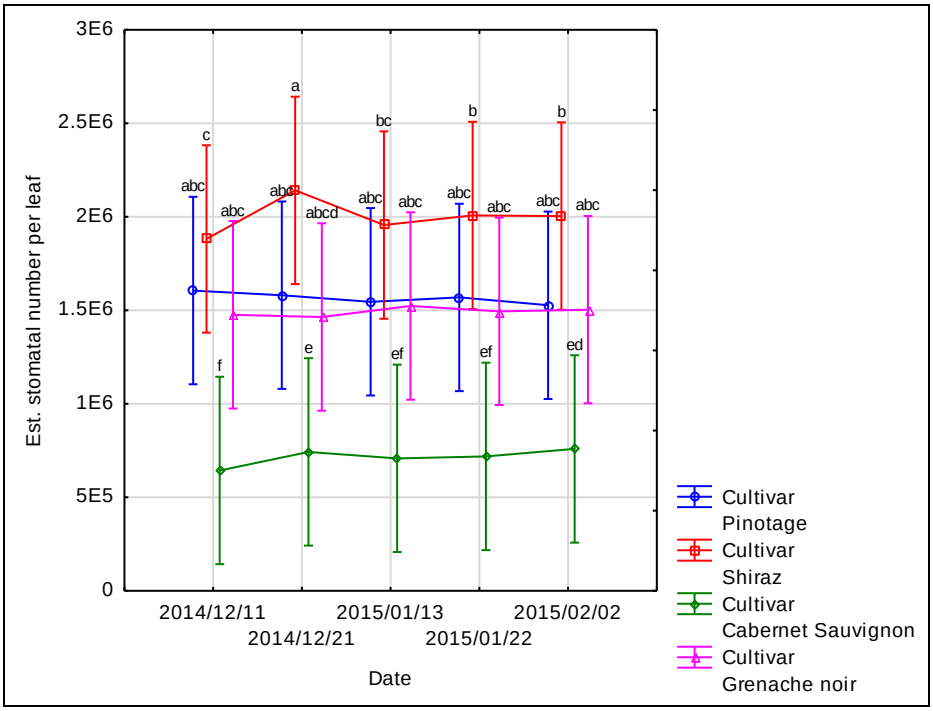

Figure 40 Mean estimated stomatal number per leaf of the different cultivars over time for the study period calculated by pooling date from all observation positions and leaf positions for each cultivar (p≤0.050); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

4.3.5.2 Leaf position x Date

The results for the interaction effect of leaf position and date on stomatal density is shown in Figure 41 and this effect was found to be significant. The stomatal density of the Apical leaves differed significantly from that of the Basal leaves for the first two dates as well as the last date. Apical 2 leaves had a higher stomatal density than all the other leaf positions. These differences were consistent over time. These results correspond to what was seen in Figure 37 with regard to leaf position. The interaction effect of leaf position and date on stomatal number per leaf was found to be very significant and the results are shown in Figure 42. The stomatal number per leaf of the Middle leaves differed significantly from that of the Basal leaves and this difference was consistent over time. The Apical and Apical 2 leaves had similar stomatal numbers per leaf and differed significantly from that of the Basal and Middle leaves. This trend was also consistent over time, but Apical 2 did not differ significantly from the Middle leaves for the second last date. These findings are comparable with those of Figure 38 with regard to leaf position.

Stellenbosch University https://scholar.sun.ac.za

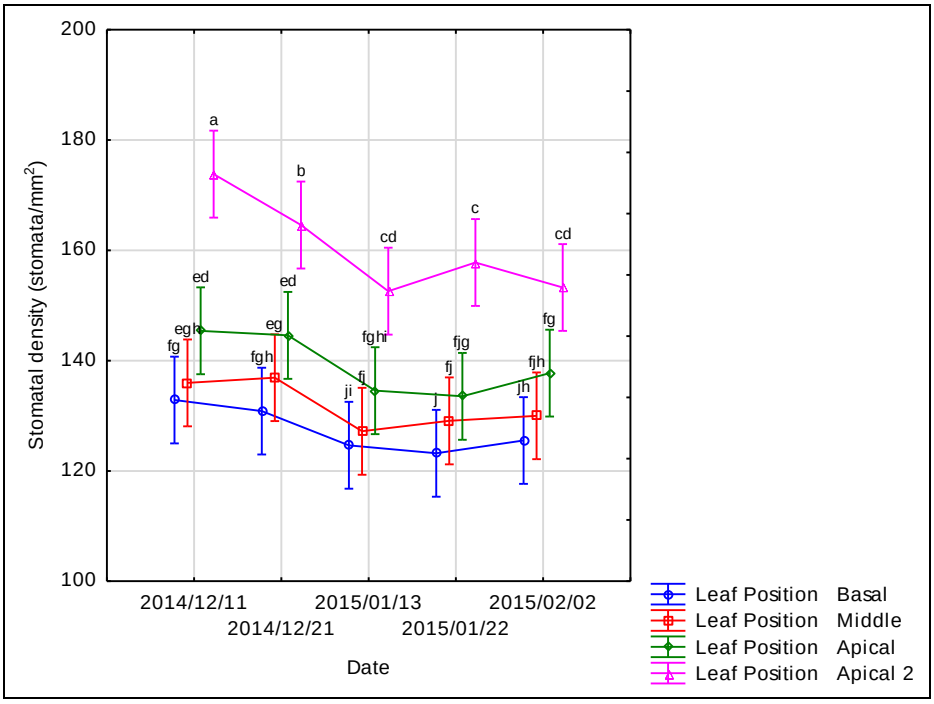

Figure 41 Mean stomatal density of different leaf positions over time for the study period calculated by pooling data from all cultivars and observation positions for each of the presented leaf positions (p≤0.05); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

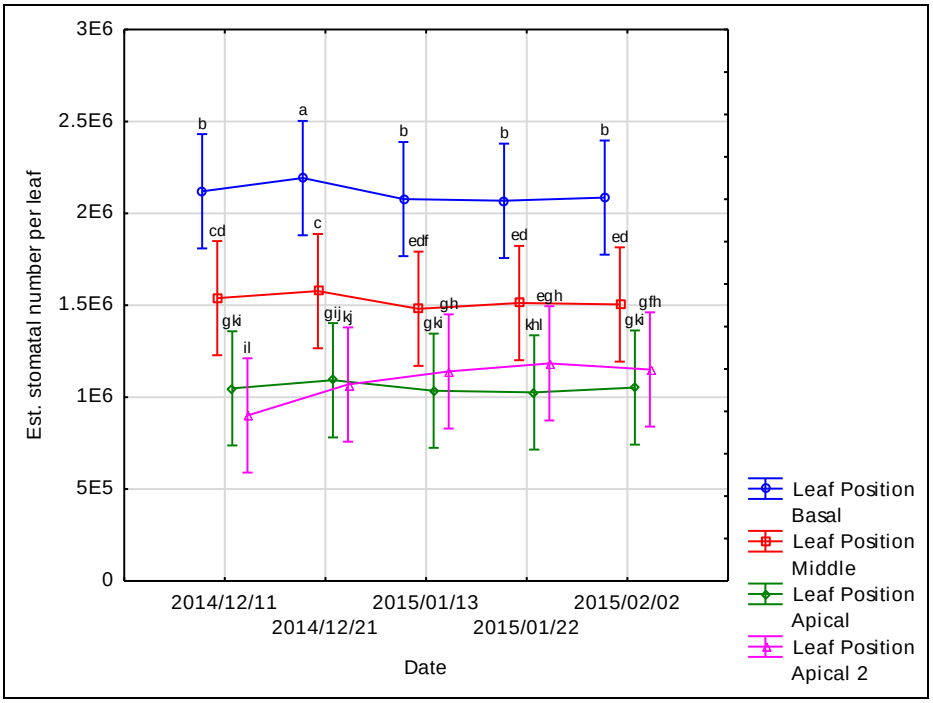

Figure 42 Mean estimated stomatal number per leaf of the different leaf positions over time for the study period calculated by pooling data from all cultivars and observation positions for the presented leaf positions (p≤0.001); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

4.3.5.3 Cultivar x Leaf position

Figure 43 indicates the interaction effect of cultivar and leaf position on stomatal density for the larger dataset in which Apical 3 was not included. The stomatal density of Apical 2 leaves differed significantly from that of the Basal and Middle leaves for all of the cultivars.

Stellenbosch University https://scholar.sun.ac.za

Furthermore, Grenache noir differed significantly from Shiraz and Cabernet Sauvignon for all leaf positions, except Apical in the case of Cabernet Sauvignon. This interaction did not have a significant effect on stomatal density (p=0.951), but when the same interaction effect was investigated for the smaller dataset including Apical 3 (Figure 44), the effect became significant (p=0.026). In this graph the differences seen in Figure 43 are not as clear. Here the only notable difference is that between Apical 3 and Apical 2 of Shiraz and Pinotage.

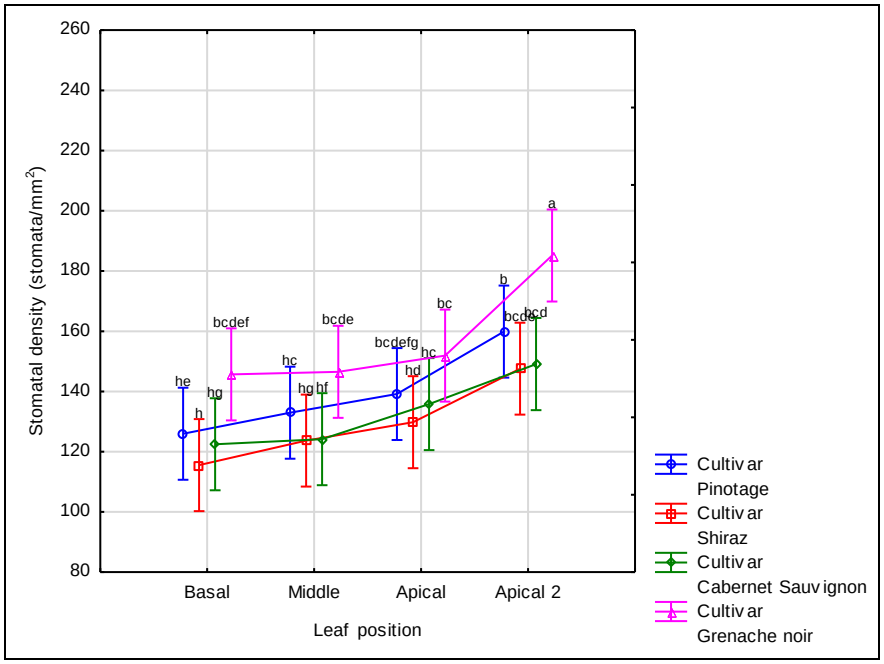

Figure 43 Mean stomatal density of the different leaf positions for each cultivar calculated by pooling data from all observation dates and observation positions for each of the presented leaf positions (p≥0.050); vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

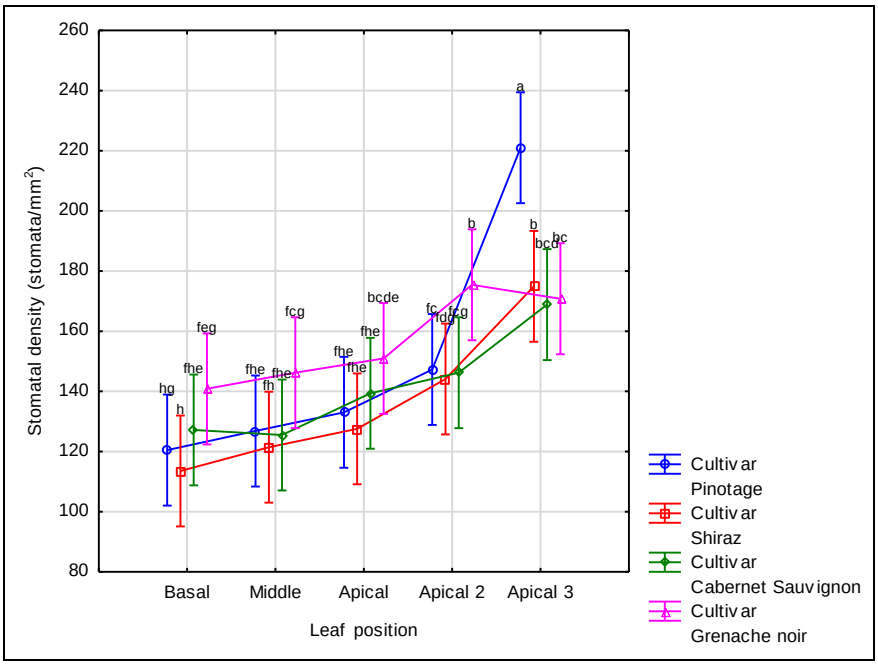

Figure 44 Mean stomatal density of the different leaf positions for each cultivar calculated by pooling data from all observation positions for each of the presented leaf positions (last observation date only) [p≤0.050]; vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

Stellenbosch University https://scholar.sun.ac.za

For stomatal number per leaf the interaction effect of cultivar and leaf position was significant in the analysis of both the larger dataset excluding Apical 3 (Figure 45), and the smaller dataset including Apical 3 (Figure 46). In both graphs the general trend was, as in Figure 36, for stomatal number per leaf to decrease as the position of the leaves moved further from the base of the shoot, except for Cabernet Sauvignon. In Figure 45 there was a significant difference between the stomatal number of Middle and Basal leaves for Shiraz, and for Pinotage differences between Basal and Middle (which were similar) and Apical. Cabernet Sauvignon showed no differences between leaf positions. In Figure 46 the stomatal number per leaf of Apical 3 differed significantly from that of Apical 2 for Shiraz and Grenache noir. Once again Cabernet Sauvignon showed no differences between leaf positions.

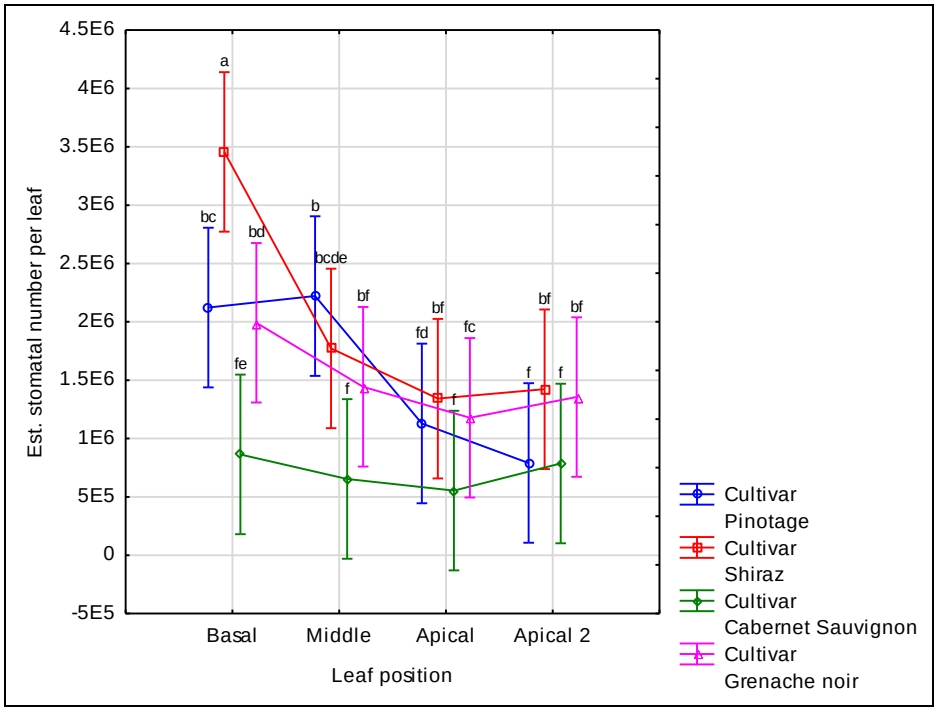

Figure 45 Mean estimated stomatal number per leaf for the different leaf positions of each cultivar calculated by pooling data from all observation dates and observation positions for the presented leaf positions ($p \leq 0.050$); vertical bars denote 95% confidence intervals. Means represented by different letter differ significanlty.

Stellenbosch University https://scholar.sun.ac.za

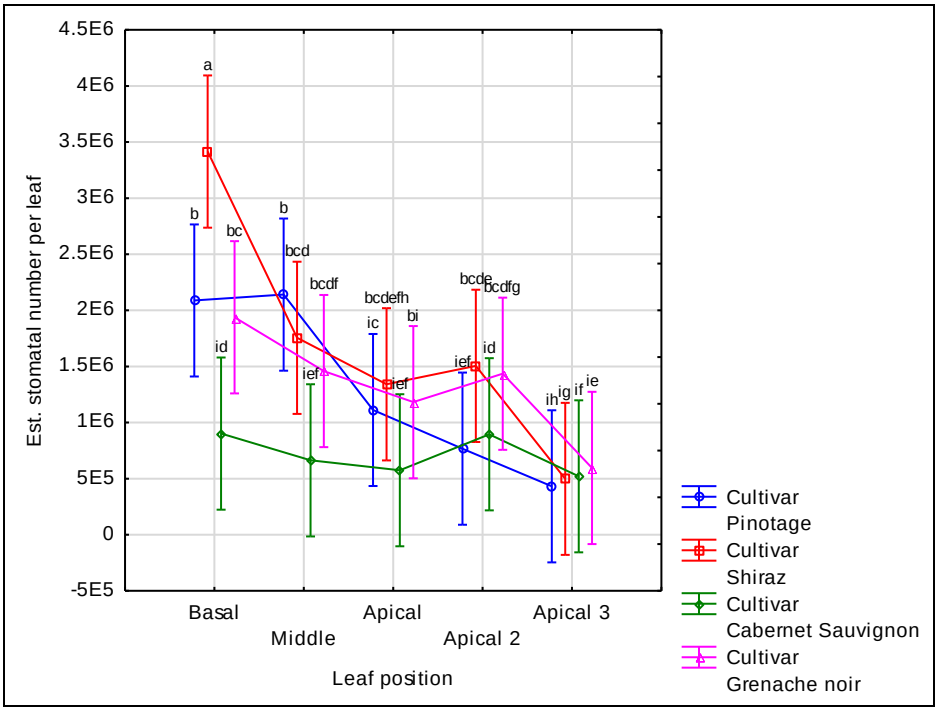

Figure 46 Mean estimated stomatal number per leaf for the different leaf positions of each cultivar calculated by pooling data from all observation positions for the presented leaf positions (last observation date only) [p≤0.050]; vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

4.3.5.4 Leaf position x Observation position

The results for the interactive effect of leaf position and observation position on stomatal density for the last date, where the Apical 3 leaves are included, is shown in Figure 47. The Basal, Middle, Apical and Apical 3 leaves showed the same trends – the stomatal density at observation positions 1 to 3 were constant and that of positions 4 to 6 were also constant, but tended to be higher than for positions 1 to 3. Apical 2 leaves displayed a different pattern. There was a significant interactive effect of leaf position and observation position on stomatal density.

Stellenbosch University https://scholar.sun.ac.za

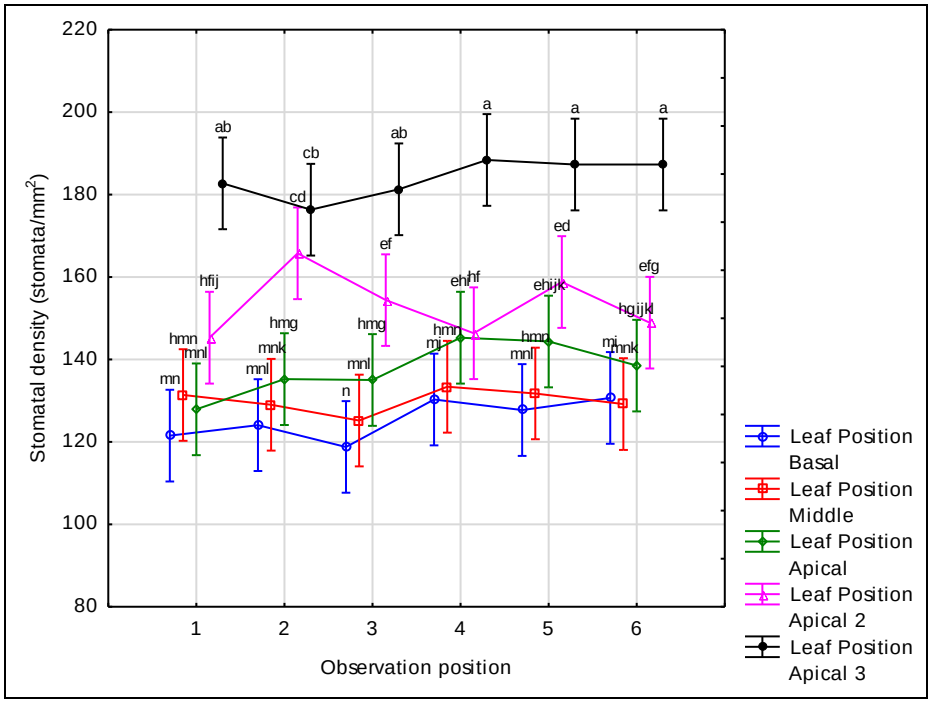

Figure 47 Mean stomatal density at each of the observation positions (1-6) for the different leaf positions calculated by pooling data from all cultivars (last observation date only) [p≤0.05]; vertical bars denote 95% confidence intervals. Means represented by different letters differ significantly.

4.3.5.5 Cultivar x Leaf position x Date

Figure 48 and Figure 49 represent the interactive effect between cultivar, leaf position and date on stomatal density and stomatal number per leaf respectively. The interactive effect on stomatal density was found to be significant. For Pinotage and Grenache noir the stomatal density of the Apical 2 leaves was consistently higher than that of the other leaf positions. The stomatal density of these same leaves was also higher on the first two dates (Pinotage) and first date (Grenache noir). In Figure 31 the first two dates showed significantly higher stomatal densities than the later dates – this could possibly have been driven predominantly by the aforementioned observations for Apical 2 of Pinotage and Grenache noir. The stomatal density of the Basal leaves of Shiraz showed a sharp increase on the second date. There were no real differences between the stomatal densities of the leaf positions for Cabernet Sauvignon. Grenache noir appeared to have higher stomatal densities for all the leaf positions compared to the other cultivars. This corresponds to the findings in Figure 33, Figure 39 and Figure 43.

Stellenbosch University https://scholar.sun.ac.za

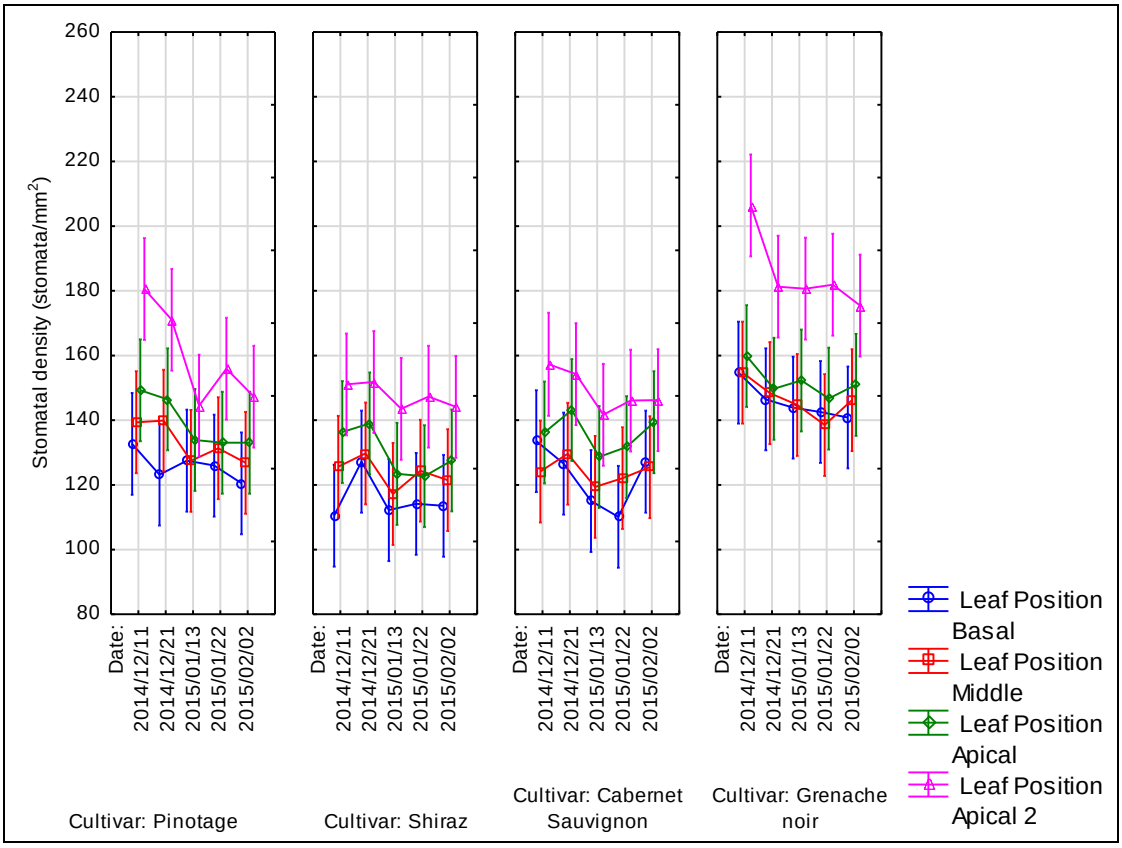

Figure 48 Mean stomatal density over the different observation dates for the different leaf positions of each cultivar calculated by pooling data from all observation positions ($p \leq 0.050$); vertical bars denote 95% confidence intervals. Note: probability letters ommitted for clarity.

There was also a significant interactive effect of cultivar, leaf position and date on stomatal number per leaf. For Pinotage the stomatal number per leaf of Basal and Middle leaves and Apical and Apical 2 was similar, as well as the former being significantly higher than the latter. The Basal leaves of Shiraz had the highest stomatal number per leaf and it peaked on the second date. This increase may have been the main driving force behind the significant increase in the stomatal number per leaf of Basal leaves (in general) on the second date as seen in Figure 42. Cabernet Sauvignon showed no significant difference between any of the leaf positions (a recurring observation). Grenache noir also showed no real difference between the leaf positions, but the values of the Basal leaves tended to be a little higher than the rest of the leaf positions. In general the stomatal number per leaf remained relatively constant over time for each cultivar's leaf position, except for the peak in the Basal leaves of Shiraz on the second date.

Stellenbosch University https://scholar.sun.ac.za

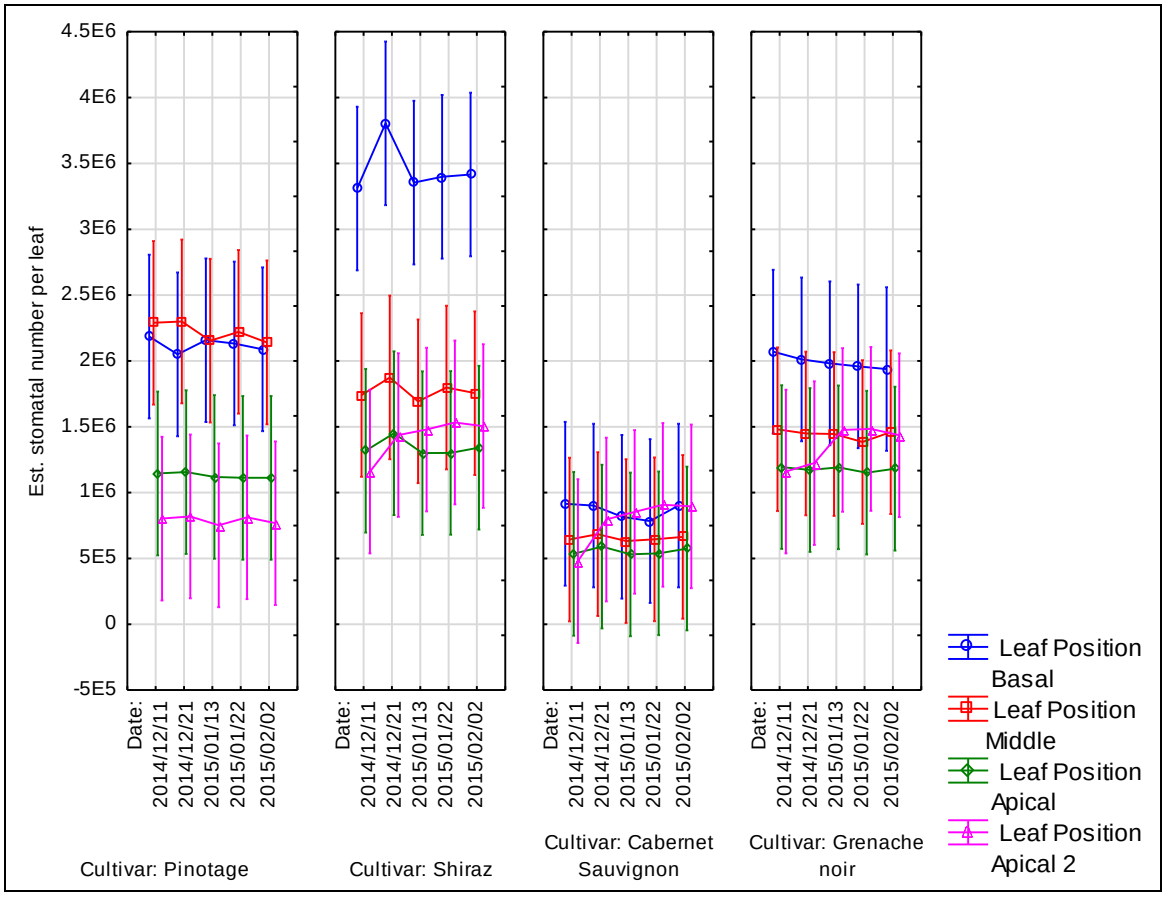

Figure 49 Mean estimated stomatal number per leaf over the different observation dates for the different leaf positions of each cultivar calculated by pooling data from all observation positions (p≤0.050); vertical bars denote 95% confidence intervals. Note: probability letters ommitted for clarity.

4.3.5.6 Cultivar x Leaf position x Observation position

The interaction effect of cultivar, leaf position and observation position on stomatal density and stomatal number per leaf is shown in Figure 50 (excluding the Apical 3 leaves) and Figure 51 (including the Apical 3 leaves). In Figure 50 no real differences are noted between the observation positions of any of the leaf positions of Pinotage, Shiraz or Cabernet Sauvignon. Apical 2 leaves did, however, tend to have a higher stomatal density than the other leaf positions for these cultivars. Grenache noir was the only cultivar which showed a significantly higher stomatal density in Apical 2 leaves, along with some notable variation between observation positions for this particular leaf position. There was a significant increase in the stomatal density at observation position 2. This increase may be responsible for the same trend noticed in the Apical 2 leaves (in general) of Figure 47. A significant interactive effect was found to be present.

From the analysis of the dataset including Apical 3 leaves, there was found to be no significant interaction effect on stomatal density. In Figure 51 the stomatal density of the Apical 3 leaves for Pinotage was the highest across all observation positions, and it also differed significantly from the other Pinotage leaf positions. In addition to this, the stomatal densities noted at observation positions 4 to 6 were also significantly higher than those of observation positions 1 to 3. For Shiraz and Cabernet Sauvignon there were no significant variations between leaf position or observation positions, although the values for Apical 3 tended to be higher than

Stellenbosch University https://scholar.sun.ac.za

those of the other leaf positions. The stomatal density of the Apical 2 leaves of Grenache noir once again showed a spike at observation position 2, as noted in Figure 50.

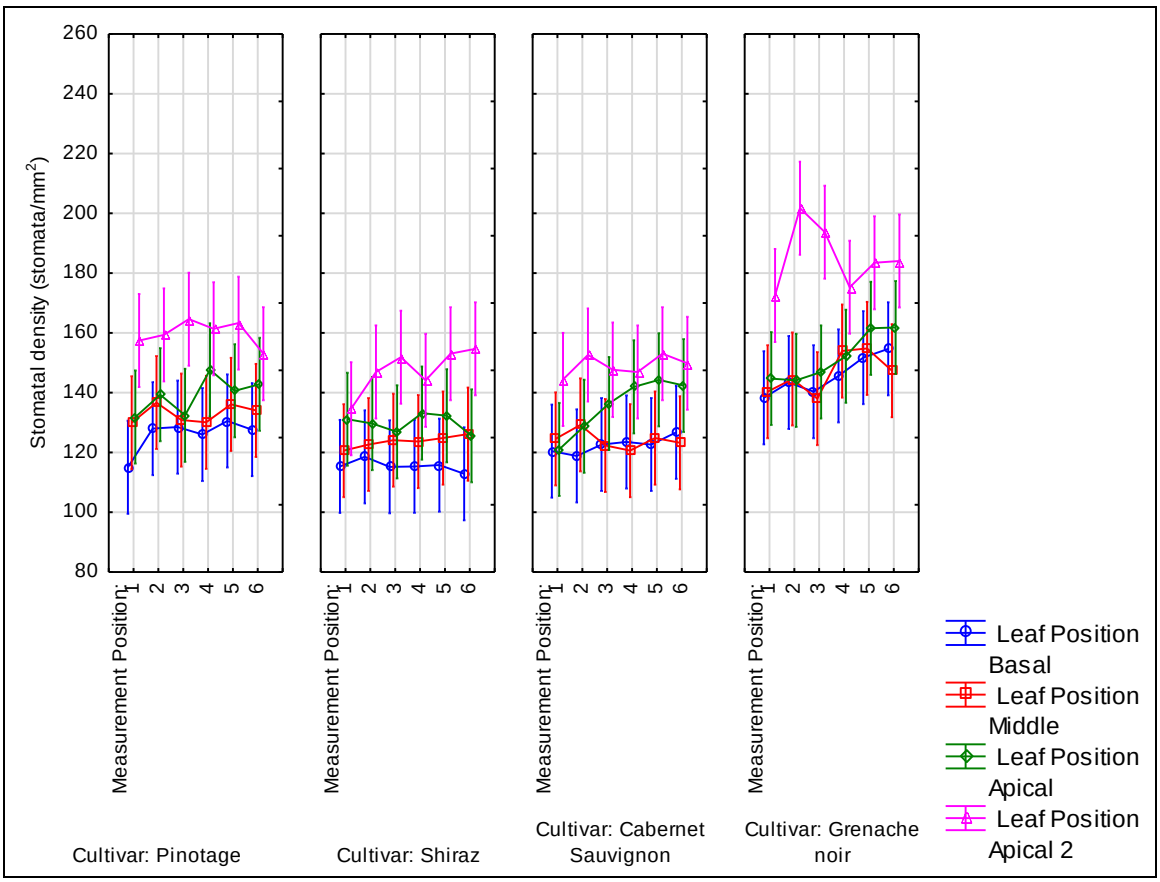

Figure 50 Mean stomatal density at each of the observation positions (1-6) for the different leaf positions of each cultivar calculated by pooling data from all observation dates ($p \leq 0.001$); vertical bars denote 95% confidence intervals. Note: probability letters ommitted for clarity.

Stellenbosch University https://scholar.sun.ac.za

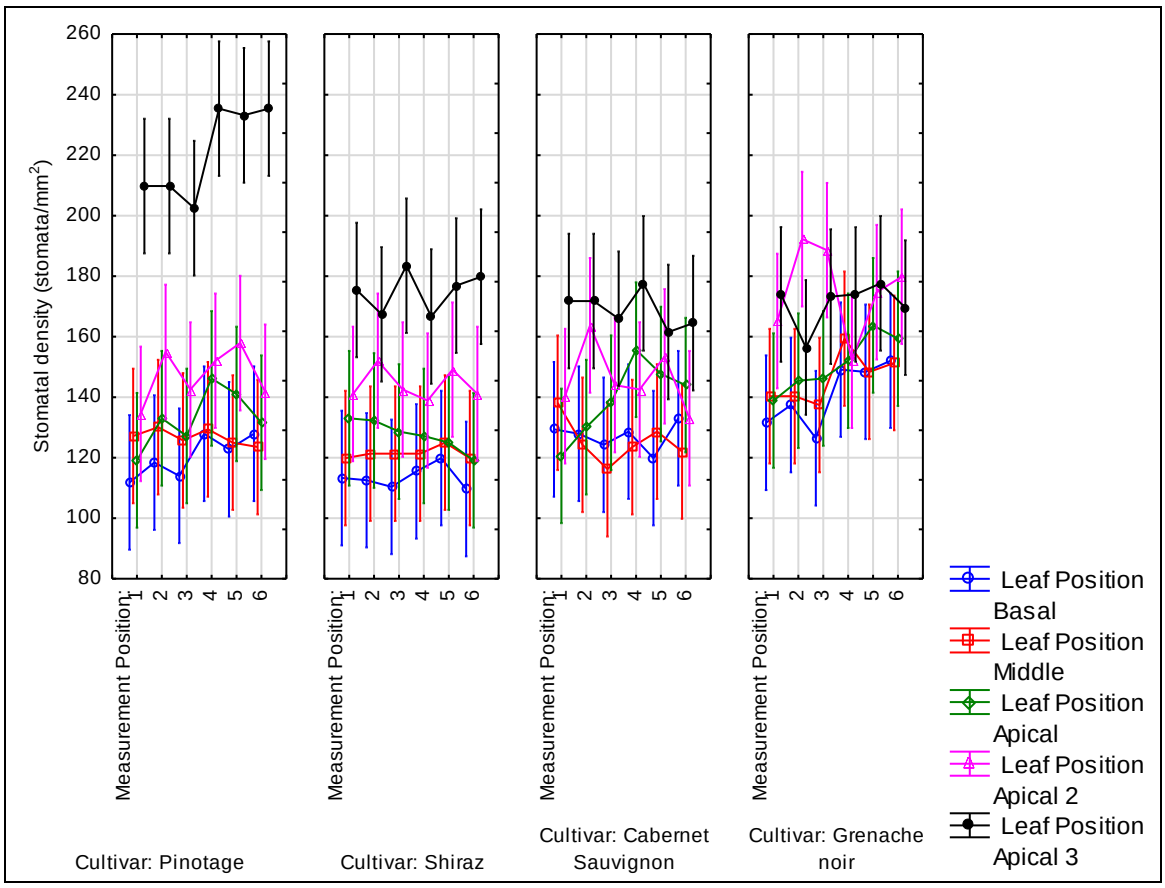

Figure 51 Mean stomatal density at each of the observation positions (1-6) for the different leaf positions of each cultivar calculated by pooling data from the last observation date only (p≥0.050); vertical bars denote 95% confidence intervals. Note: probability letters ommitted for clarity.

4.4 Discussion

4.4.1 Stomatal density - observations over time

The main effect of date showed differences in stomatal density, with the first two dates generally having higher values (Figure 31). The same observation was made for Apical 2 leaves in Figure 41, which represented the interaction effect of leaf position and date. Since stomatal density is the number of stomata per unit area, it is logical that it will be affected by the number of cells (stomatal and epidermal) within an area and also the size of these cells. Stomatal density would therefore vary throughout the leaf development process, and this assumption was made when explaining some of the trends observed. Furthermore, the formation of stomata is a complicated, carefully regulated process with only certain epidermal cells undergoing the transition to stomatal guard cells. Leaf development in grapevines consists of a series of stages starting with the initial emergence of the leaf. This is then followed by unfolding, rapid expansion of the leaf blade and eventually senescence (Kriedemann *et al.*, 1970). The development of leaves involves different processes related to cell cycling, which determines leaf morphogenesis, the tissue-specific cell division patterning and cell differentiation. These processes all occur simultaneously during leaf growth (Donnelly *et al.*, 1999). The expansion of the leaf blade occurs mainly through cell division and cell enlargement. During cell division and differentiation, new cells are formed and their fates determined, while cell enlargement allows growth only by the increase in size of existing cells (Keller, 2010). The cell division phase of grapevine leaves stops when the leaves have reached about half of their full size (Keller, 2010).

Stellenbosch University https://scholar.sun.ac.za

Stomata are formed while cell division and cell differentiation occurs and thus no new stomata will be formed once the final cell enlargement phase begins. This coincides with the observation of Tichá (1982) that stomata are formed until leaves reach 10 to 50% of their full size (results from various plant species). During this latter enlargement phase it would thus be possible for stomatal density to decrease as the cells enlarge taking up more space and thus lowering the number of cells per unit area. Grapevine shoots and leaves are known to have a long growth period spanning from flowering until post-véraison (Palliotti *et al.*, 2000), but it must be noted that leaves reach their full size 30 to 40 days after unfolding (Pratt, 1974). The first two measurement dates in this study occurred between pea-size and bunch closure and the last three dates between pre- and post-véraison. An estimated phenological progression of the subject vines is presented in Table 18.

Table 18 Estimated phenological progression for the investigated vines over the period of measurements.

	Pinotage	**Shiraz**	**Cabernet Sauvignon**	**Grenache noir**
11/12/2014	Bunch closure	Bunch closure	Bunch closure	Pea-size
21/12/2014	Bunch closure	Bunch closure	Bunch closure	Bunch closure
13/01/2015	Pre-véraison	Pre-véraison	Pre-véraison	Pre-véraison
22/01/2015	Véraison	Véraison	Pre-véraison	Pre-véraison
02/02/2015	Post-véraison	Post-véraison	Véraison	Véraison

It can therefore be assumed that the younger leaves were in the cell division phase during the first two dates and then in a cell enlargement phase during the later dates. This may explain the decreased and constant stomatal density in the later observations. Miyazawa *et al.* (2006) noted that an increase in leaf area will lead to a decrease in stomatal density if there is not a simultaneous increase in stomatal number (no more stomata formed). Judging by the L1 length progression (Figure 52) the leaves did not appear to enlarge during the latter period of the study. However, leaf growth is not achieved only in a lengthwise direction, but through intercalary growth between the veins as well (Pratt, 1974; Van Lijsebettens & Clarke, 1998; Keller, 2010). Thus, the leaves could have expanded width-wise and within the lobes - this would not be represented by the L1 length.

4.4.2 Stomatal density - observations between cultivars

When the main cultivar effect was investigated, some of the cultivars were found to differ from the others. While Pinotage, Shiraz and Cabernet Sauvignon had very similar stomatal densities, Grenache noir had a significantly higher density (Figure 33). This same trend in stomatal density between cultivars was also noticed in Figure 39, Figure 41, Figure 48 and Figure 50, which represented results of various interaction effects of cultivar and other factors. Since stomatal development is a process which is closely guided on a molecular level, it can be proposed that stomatal density is genetically predetermined for different cultivars. This is further supported by the fact that even in combination with other factors, the cultivar effect still held true. Cultivar differences with regard to stomatal density have also been documented by Palliotti *et al.* (2000) who found that, in general, Trebbiano Toscano had a higher stomatal density than Cabernet franc. Düring (1980) also determined the stomatal densities of different *Vitis* species and cultivars. Table 19 represents stomatal density values as found in literature.

Stellenbosch University https://scholar.sun.ac.za

Table 19 Stomatal density of different *Vitis* species and cultivars as reported in literature.

Species and cultivar	Rootstock	Experiment setup - field or pot (F/P)	Growth conditions	Mean stomatal density (number/mm^2)	Reference
Vitis vinifera cv. Forta	Own roots	F	Standard*	236.8	(Düring, 1980)
Vitis vinifera cv. Optima	Own roots	F	Standard*	249.0	
Vitis vinifera cv. Riesling	Own roots	F	Standard*	200.8	
Vitis vinifera cv. Riesling	Own roots	P	Glasshouse	149.8	
Vitis amurensis	Own roots	F	Standard*	278.9	
Vitis berlandieri	Own roots	F	Standard*	256.1	
Vitis cinerea	Own roots	F	Standard*	362.6	
Vitis rupestris	Own roots	F	Standard*	175.5	
Vitis vinifera cv. Cabernet franc	Kober 5BB	F	Sun & Shade	160.3	(Palliotti *et al.* , 2000)
Vitis vinifera cv. Trebbiano Toscano	Kober 5BB	F	Sun & Shade	205.6	
Vitis vinifera cv. Cabernet Sauvignon	Own roots	F	Standard*	180.0	(Rogiers *et al.* , 2009)
Vitis vinifera cv. Chardonnay	Own roots	F	Standard*	225.5	
Vitis vinifera cv. Pinot noir	Own roots	F	Standard*	184.0	
Vitis vinifera cv. Shiraz	Own roots	F	Standard*	167.4	
Vitis vinifera cv. Chardonnay	Not specified	P	Glasshouse: Ambient CO_2	180.0	(Rogiers *et al.* , 2011)
Vitis vinifera cv. Pinotage	1103 Paulsen	F	Water-stressed & Sun	119.1	(Serra, 2014)
Vitis vinifera cv. Pinotage	1103 Paulsen	F	Water-stressed & Shade	91.0	
Vitis vinifera cv. Pinotage	140 Ruggeri	F	Water-stressed & Sun	113.8	
Vitis vinifera cv. Pinotage	140 Ruggeri	F	Water-stressed & Shade	96.3	
Vitis vinifera cv. Pinotage	99 Richter	F	Standard*	139.5	This study
Vitis vinifera cv. Shiraz	99 Richter	F	Standard*	129.1	
Vitis vinifera cv. Cabernet Sauvignon	99 Richter	F	Standard*	132.9	
Vitis vinifera cv. Grenache noir	99 Richter	F	Standard*	157.3	

*atmospheric conditions

Stellenbosch University https://scholar.sun.ac.za

4.4.3 Stomatal density - observations between different leaf positions

The main effect of leaf position also had a significant effect on stomatal density and this was reported in Figure 35 and Figure 37. In both of these graphs Basal, Middle and Apical leaves did not differ with regard to stomatal density, but Apical 2 and Apical 3 leaves differed significantly from one another as well as from the other leaf positions. This trend was also noticed in Figure 41 for the leaf position and date interaction effect. For the leaf position and cultivar interaction effect, however, the difference in stomatal density for these younger leaves was not as clear.

Revisiting the hypothesis of leaf growth stated earlier, this increase in stomatal density for the youngest leaves (namely Apical 2 and Apical 3), may be explained by the fact that they had the shortest growth period during the study. This could possibly lead to the average cell size being smaller for these leaves and thus an increased stomatal density would be expected. If the leaf cells were also still actively dividing and differentiating during this time, thereby producing new stomata, this could further increase stomatal density due to the limited leaf size over which to spread the newly forming cells. It is also known that mature leaves may signal younger, developing leaves to alter their stomatal density in response to an environmental stimulus, such as increased environmental CO_2 content or shady conditions (Lake *et al.*, 2001; Miyazawa *et al.*, 2006). Since the vineyard in which the study was conducted was cultivated under dryland conditions, water deficits may have occurred during the course of the growing season. Moderate water stress has been found to positively influence stomatal density (Xu & Zhou, 2008). It could therefore be possible that the younger leaves, which were expanding during this time, altered their stomatal density in response to moderate water stress, if present. The increase in stomatal density for leaves closer to the shoot tip could also be explained by sun exposure and this is perhaps the most likely reason. These leaves are often fully exposed, whereas leaves lower down in the canopy will be subjected to varying degrees of shade due the crowding of the canopy by surrounding leaves. The sides of a trellised canopy are also not exposed to light for the entire day, but the top of the canopy row (where the most apical leaves are found) will receive irradiance for the entire length of day if unobstructed by windbreaks or other structures. Shaded leaves have been found to have a lower stomatal density than exposed leaves (Palliotti *et al.*, 2000; Serra, 2014) and this could therefore explain the findings in this study. In the study by Düring (1980) the stomatal density of mature and young leaves were also investigated – interestingly no differences were found for *Vitis vinifera,* but for other *Vitis* species.

4.4.4 Stomatal density - observations between different measurement positions on the leaf

In the studies by Düring (1980) and Palliotti *et al.* (2000), the on-leaf variation of stomatal density was also investigated. Düring (1980) looked at positions next to the main vein, centre of the lobe and tip of the leaf, and Palliotti *et al.* (2000) investigated positions on the basal, middle and tip lobes. Neither study found significant differences between the stomatal densities at the various positions. In this study, however, there were differences between on-leaf positions for Apical 2 and Apical 3 leaves (Figure 46 and Figure 47). This could possibly be explained by the fact that these younger leaves were still expanding and that the expansion between the veins at different areas of the leaves were not the same. (Bodor *et al.*, 2012) states that leaves differ in their characteristics such as leaf area, vein length, vein ratios (central to lateral), as well as the

Stellenbosch University https://scholar.sun.ac.za

angles between the veins. These characteristics differ between cultivars, forming the basis for ampelometric identification, but they should also differ during the leaf development phase for a particular leaf. It is not clear whether the leaves used by Palliotti *et al.* (2000) were mature or young, but Düring (1980) used both mature and young leaves and his findings are thus contradictory to what was found this study. The differences found do not appear to apply to all young leaves of all cultivars (Figure 50 and Figure 51), therefore it could be possible that the differences noted in Figure 47 were due to the interactive effects between different factors in these investigations.

4.4.5 Stomatal number per leaf - observations over time

The other variable investigated in this study was stomatal number per leaf. The number of stomata per leaf was estimated by multiplying the measured stomatal density from the various observations by the relevant leaf area. Being a non-destructive study, leaf area had to be estimated from the L1 length of the leaves – this process was described in Chapter 3 of this thesis. Stomatal number per leaf is thus dependent on both stomatal density and leaf size.

The main effect of date also had a significant effect on stomatal number per leaf (Figure 32) with the general stomatal number per leaf on the second date varying significantly from that on the first and third dates. The limited variation in stomatal number per leaf over time can be ascribed to the fact that the L1 length of leaves did not change significantly during the period for which the data was reported. Figure 52 shows the average L1 length progression of the various leaf position leaves – the arrow line denotes the start of the period relevant to the results. The L1 lengths did, however, increase slightly from the first to the second observation date – this coincides with the increase in stomatal number per leaf for this date.

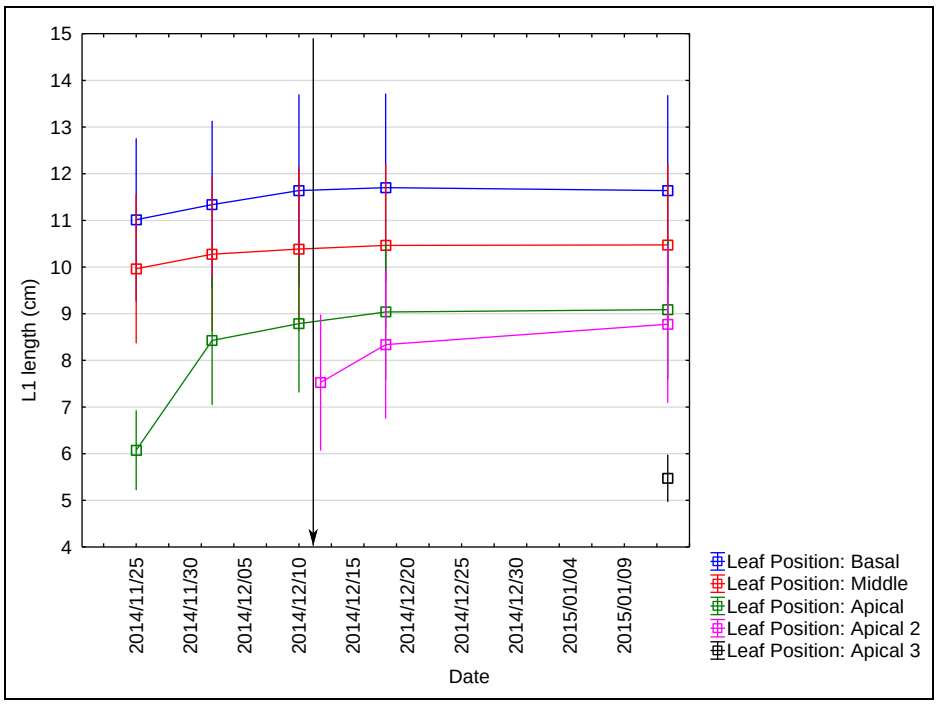

Figure 52 Average leaf main vein length (L1) over time for the different leaf positions. The arrow denotes the start of the study period; vertical bars denote 95% confidence intervals.

Stellenbosch University https://scholar.sun.ac.za

4.4.6 Stomatal number per leaf - observations between cultivars

As with stomatal density, some cultivars differed with regard to stomatal number per leaf (Figure 34), with a significant difference being observed between Shiraz and Cabernet Sauvignon. The mean L1 (leaf main vein) lengths (all measurements for all the leaves) for the different cultivars are shown in Figure 53. Cabernet Sauvignon had the smallest leaves on average (based on the L1 length) while the other cultivars all had similar sized leaves. This corresponds with the observations that Cabernet Sauvignon had the least stomata per, and that Pinotage, Shiraz and Grenache noir had similar sized leaves and also similar stomatal numbers per leaf. The difference between stomatal number per leaf in Shiraz and Cabernet Sauvignon is also noticed when various interaction effects with cultivar, as one of the interacting factors, were investigated – refer to Figure 40, Figure 45 and Figure 46. In the latter two figures, the differences in stomatal number per leaf between Shiraz and Cabernet Sauvignon are particularly prominent for Basal and Middle leaves. The high number of stomata observed for Basal leaves of Shiraz may also lead to the large number of stomata per leaf for this cultivar in general (Figure 34).

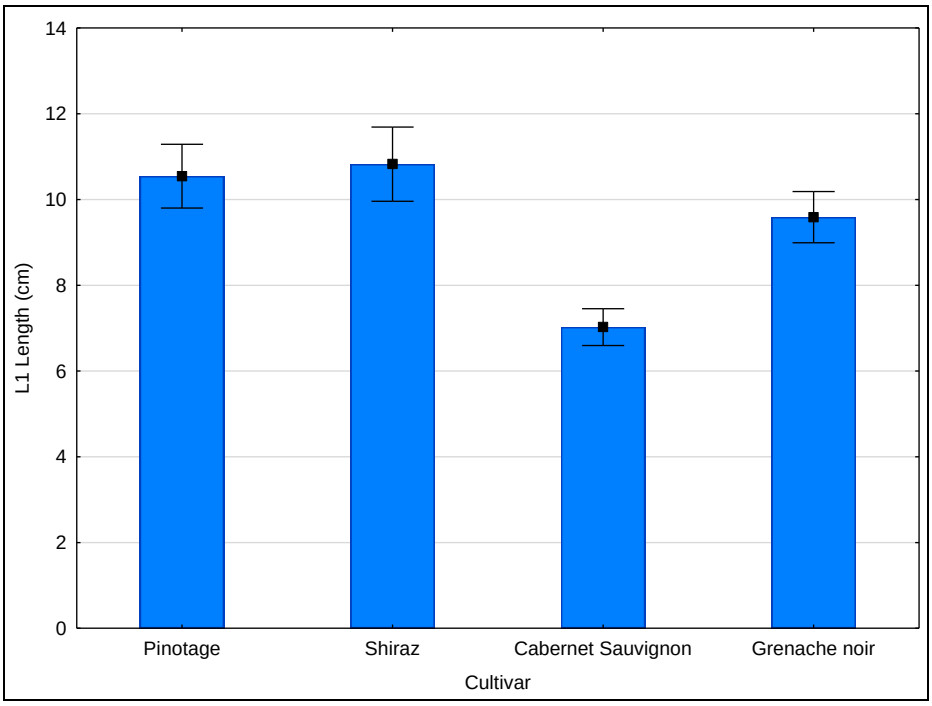

Figure 53 Mean L1 (main central vein) lengths of the leaves for the different cultivars; vertical bars denote 95% confidence intervals.

4.4.7 Stomatal number per leaf - observations between different leaf positions

A difference was also found in stomatal number per leaf between the different leaf positions. These differences were shown in Figure 36 (including Apical 3 leaves) and Figure 38 (excluding Apical 3 leaves). The trends can be explained from the average L1 lengths of the different leaf positions (Figure 54). Apical and Apical 2 leaves had very similar sized leaves (similar L1 lengths) corresponding to the similar stomatal number per leaf observed for these two leaf positions. The Basal leaves were the largest, also having the largest number of stomata and Apical 3 leaves being the smallest, had the least number of stomata. Therefore, even though stomatal density increased for leaves in the direction of the shoot tip, the smaller leaf sizes in this direction led to the decrease in stomatal number per leaf being noted. This leads to the

Stellenbosch University https://scholar.sun.ac.za

assumption that leaf size, and not stomatal density, is dominant in determining stomatal number per leaf.

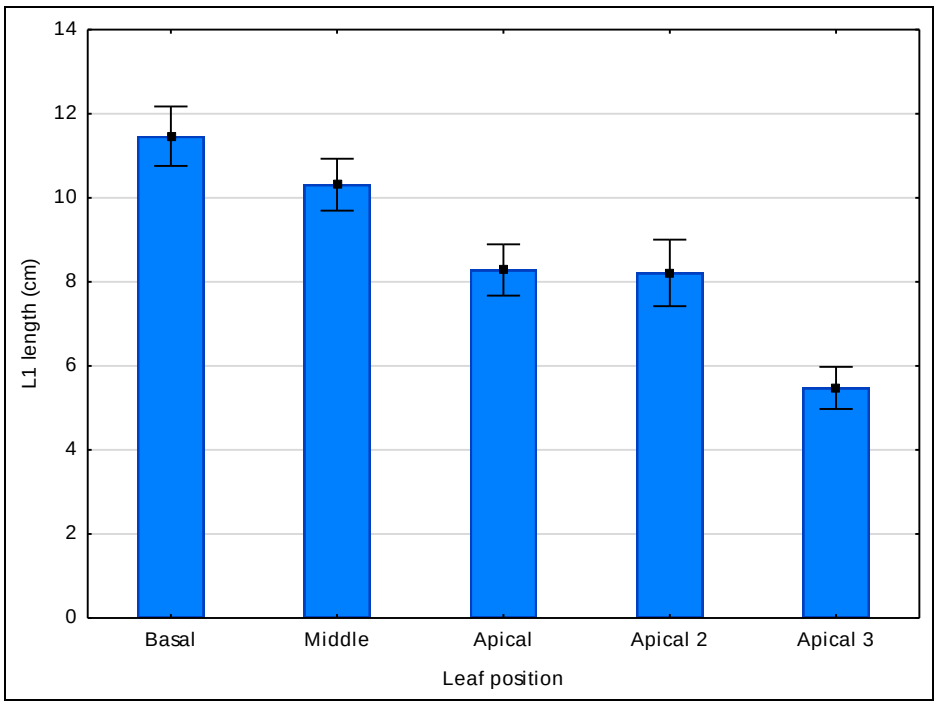

Figure 54 Mean L1 (main central vein) lengths of the different leaf positions; vertical bars denote 95% confidence intervals.

When the interactive effect of cultivar and leaf position on stomatal number per leaf was investigated (Figure 45), all leaf positions of Cabernet Sauvignon had similar stomatal numbers. This same trend is noted in Figure 49 in which the interactive effect of cultivar, leaf position and date was investigated. Comparing the mean L1 lengths of the leaf positions per cultivar (Figure 55), Cabernet Sauvignon stood out as having leaves that were similar in size across all leaf positions. The Basal and Middle leaves of Pinotage were also similar in size, once again explaining the deviation from the general trend regarding stomatal number per leaf observed in Figure 45 and Figure 46. The large confidence intervals observed for the Apical 3 leaves in Figure 55 can be explained by the fact that these leaves had fewer measurements than the other leaf position leaves, and therefore less data available from which a mean was calculated.

Stellenbosch University https://scholar.sun.ac.za

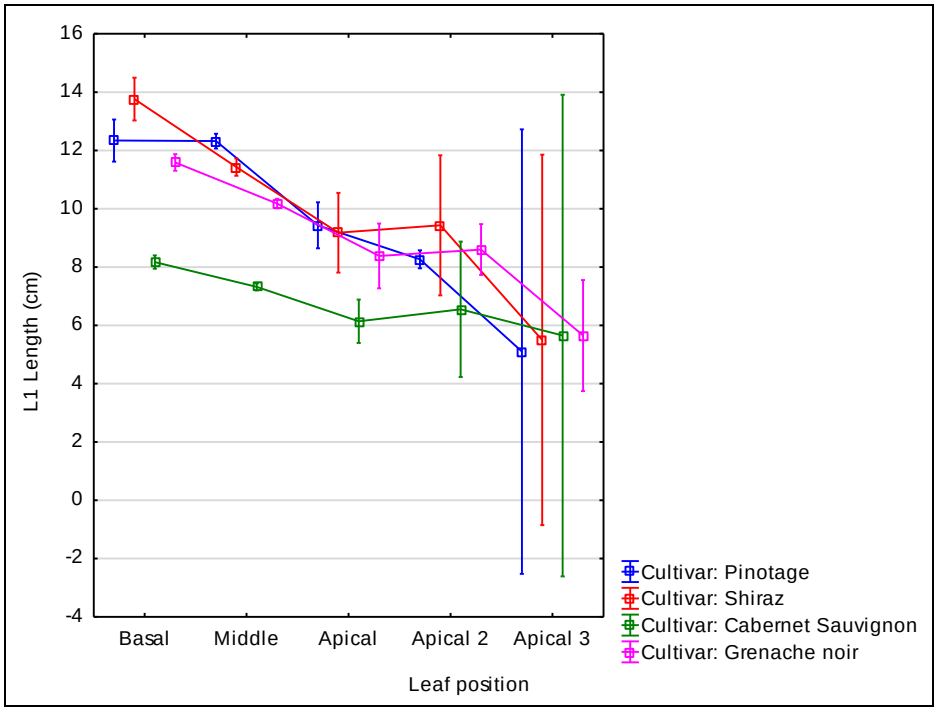

Figure 55 Average L1 (main central vein) length of the different leaf positions for the four cultivars.

4.5 Conclusion

It is clear from the results obtained from this study that there are many factors at play that determine stomatal density. Stomatal development is a complex process and some effects cannot be easily defined with simple explanations, but rather through a series of possibilities. When factors are investigated in combination, they may either bring about effects that are similar to what was seen for the individual main effects, or the results can differ. The stomatal number per leaf appears to be determined predominantly by leaf size and to a lesser extent by stomatal density – a large leaf with a low stomatal density can have more stomata per leaf than a smaller leaf with a higher stomatal density.

In this study, stomatal density and number per leaf did not appear to vary much over time, although the main effect of date was found to be significant. For stomatal density, the first two dates differed from the later dates, and only the second date varied in the case of stomatal number per leaf. If, however, the study was conducted from an earlier stage (starting prior to 11 December) the effect over time may have been more significant.

The main effect of cultivar had a significant influence on determining stomatal density, but not stomatal number per leaf even though there were differences between some cultivars. This means that cultivars may not always differ from one another with regard to their stomatal stomatal numbers per leaf, but it is a possibility and totally dependent on the cultivars investigated. In other words, other cultivars not investigated in this study may have reacted differently with regards to stomatal number per leaf. The reported cultivar differences were seen not only in the main effect investigation, but also for various interactive effects. Such interactions include those of cultivar and date (Figure 39), and cultivar and leaf position (Figure 43 and Figure 44). This suggests that the stomatal density of cultivars is genetically predetermined and that the impact of additional factors lie within the “boundaries” set by this inherent determination of stomatal density. An example of this is the fact that Grenache noir consistently had a higher stomatal density than the other three cultivars. The type of leaf, with regard to the level of sun

Stellenbosch University https://scholar.sun.ac.za

exposure, which is selected for investigations is also important, since sun exposed leaves have been found to have higher stomatal densities (Palliotti *et al.*, 2000; Serra *et al.*, 2014).

Stomatal density tended to increase in the direction of the shoot tip, while stomatal number per leaf decreased due to the smaller leaf sizes of the younger leaves. Basal, Middle and Apical leaves did not differ significantly in their stomatal density, but the differences were significant in Apical 2 and Apical 3 leaves. The most probable explanation for this increase in stomatal density is the fact that leaves closer to the shoot tip are usually fully exposed and receive sunlight for a greater period of the day, than leaves that are closer to the base of the shoot. Another likely explanation is the fact that these younger leaves had a shorter growth period during the study than the older leaves (Basal, Middle and Apical). The cells are thus expected to be smaller with more cells occupying a specific unit of leaf area. This will lead to an increase in stomatal density and especially if there was still cell division and differentiation taking place, allowing for new stomata to be formed. The variation between stomatal densities at different positions on a leaf was determined by the leaf position – significant differences were only observed for the younger, smaller leaves. In other studies (Düring, 1980; Palliotti *et al.*, 2000) this was not confirmed and there is thus some controversy regarding these findings.

From a physiological perspective, the effect of stomatal density is mostly related to stomatal conductance, and particularly in determining the maximum rate of gaseous exchange possible for the amount of water that is available to the plant (balancing CO_2 assimilation with transpiration). It is thus logical that stomata will aim at increasing the water use efficiency (WUE) of a plant (Wang *et al.*, 2007; Xu & Zhou, 2008). A cultivar's susceptibility or resistance to drought may thus be explained by investigating stomatal density, size and function. Grapevine cultivars are classified as being near-isohydric or -anisohydric based on their ability to regulate their plant water status under conditions of drought. Grenache noir and Cabernet Sauvignon have both been classified as near-isohydric, meaning that they are capable of regulating their water status (Schultz, 2003; Soar *et al.*, 2006; Tramontini *et al.*, 2014). Shiraz on the other hand, is near-anisohydric with almost no ability to regulate plant water status – cultivars such as these are unable to maintain a constant stem water potential and will suffer under drought conditions (Schultz, 2003; Soar *et al.*, 2006). What is interesting from the findings of this study, is that the stomatal number per leaf of Shiraz and Cabernet Sauvignon are similar while behaving differently under drought conditions. It is thus clear that the regulation of plant water status is not necessarily related to stomatal number, but rather stomatal function. This has also been stated by Schultz (2003).

In a study by Tanaka *et al.* (2013) the effect of stomatal density on photosynthetic capacity in *Arabidopsis thaliana* was investigated. Their findings proved that an increase in stomatal density positively affected the rate of photosynthesis through an increase in gaseous exchange, and not through increased carboxylation. In conjunction to this, an increase in transpiration was also noted. Candolfi-Vasconcelos *et al.* (1994) states that the photosynthetic rate of the leaves at the top of the canopy is higher because young, fully expanded leaves photosynthesise more than older leaves. This could be due to the increased stomatal density of these leaves as noted in this study, suggesting that the findings of Tanaka *et al.* (2013) may be true for grapevines as well.

Stellenbosch University https://scholar.sun.ac.za

4.6 Literature cited

Arve, L.E., Torre, S., Olsen, J.E. & Tanino, K.K., 2011. Stomatal Responses to Drought Stress and Air Humidity, Abiotic Stress in Plants - Mechanisms and Adaptations. Shanker, A. (ed). InTech.

Assmann, S.M., 1988. Enhancement of the stomatal response to blue light by red light, reduced intercellular concentrations of carbon dioxide, and low vapor pressure differences. Plant Physiol. 87(1), 226–231.

Assmann, S.M. & Shimazaki, K-I., 1999. The multisensory guard cell. Stomatal responses to blue light and abscisic acid. Plant Physiol. 119(3), 809–815.

Bodor, P., Baranyai, L., Balo, B., Toth, E., Strever, A.E., Hunter, J.J. & Bisztray, G.D., 2012. GRA.LE.D (GRApevine LEaf Digitalization) Software for the detection and graphic reconstruction of ampelometric differences between *Vitis* leaves. S. Afr. J. Enol. Vitic. 33(1), 1–6.

Candolfi-Vasconcelos, M.C., Koblet, W., Howell, G.S. & Zweifel, W., 1994. Influence of defoliation, rootstock, training system, and leaf position on gas exchange of Pinot noir grapevines. Am. J. Enol. Vitic. 45(2), 173–180.

Casson, S.A. & Gray, J.E., 2008. Influence of environmental factors on stomatal development. New Phytol. 178(1), 9–23.

Casson, S.A. & Hetherington, A.M., 2010. Environmental regulation of stomatal development. Curr. Opin. Plant Biol. 13(1), 90–95.

Donnelly, P.M., Bonetta, D., Tsukaya, H., Dengler, R.E. & Dengler, N.G., 1999. Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. Dev. Biol. 215(2), 407–419.

Düring, H., 1980. Stomatafrequenz bei Blättern von *Vitis*-Arten und -Sorten. Vitis 19, 91–98.

Ireland, C.R., 2010. Experimental Statistics for Agriculture and Horticulture. CABI, Cambridge.

Keller, M., 2010. The Science of Grapevines: Anatomy and Physiology. Academic Press, Amsterdam.

Kriedemann, P.E., Kliewer, W.M. & Harris, J.M., 1970. Leaf age and photosynthesis in Vitis vinifera L. Vitis 9, 97–104.

Lake, J.A., Quick, W.P., Beerling, D.J. & Woodward, F.I., 2001. Signals from mature to new leaves. Nature 411(6834), 154.

Van Lijsebettens, M. & Clarke, J., 1998. Leaf development in *Arabidopsis*. Plant Physiol. Biochem. 36(1-2), 47–60.

Miyazawa, S-I., Livingston, N.J. & Turpin, D.H., 2006. Stomatal development in new leaves is related to the stomatal conductance of mature leaves in poplar (*Populus trichocarpa* x *P. deltoides*). J. Exp. Bot. 57(2), 373–380.

Palliotti, A., Cartechini, A. & Ferranti, F., 2000. Morpho-anatomical and physiological characteristics of primary and lateral shoot leaves of Cabernet Franc and Trebbiano Toscano grapevines under two irradiance regimes. Am. J. Enol. Vitic. 51(2), 122–130.

Pillitteri, L.J. & Torii, K.U., 2012. Mechanisms of stomatal development. Annu. Rev. Plant Biol. 63, 591–614.

Pratt, C., 1974. Vegetative anatomy of cultivated grapes - a review. Am. J. Enol. Vitic. 25(3), 131–150.

Rogiers, S.Y., Hardie, W.J. & Smith, J.P., 2011. Stomatal density of grapevine leaves (*Vitis vinifera* L.) responds to soil temperature and atmospheric carbon dioxide. Aust. J. Grape Wine Res. 17(2), 147–152.

Stellenbosch University https://scholar.sun.ac.za

Schultz, H.R., 2003. Differences in hydraulic architecture account for near-isohydric and anisohydric behaviour of two field-grown *Vitis vinifera* L. cultivars during drought. Plant Cell Environ. 26(8), 1393–1405.

Serra, I., 2014. Grapevine (*Vitis vinifera* L., cv. Pinotage) responses to water deficit modulated by rootstocks. PhD Dissertation, Stellenbosch University, Private Bag X1, 7602, Matieland (Stellenbosch), South Africa.

Serra, I., Strever, A., Myburgh, P.A. & Deloire, A., 2014. Review: the interaction between rootstocks and cultivars (*Vitis vinifera* L.) to enhance drought tolerance in grapevine. Aust. J. Grape Wine Res. 20(1), 1–14.

Soar, C.J., Speirs, J., Maffei, S.M., Penrose, A.B., McCarthy, M.G. & Loveys, B.R., 2006. Grape vine varieties Shiraz and Grenache differ in their stomatal response to VPD: apparent links with ABA physiology and gene expression in leaf tissue. Aust. J. Grape Wine Res. 12(1), 2–12.

Tanaka, Y., Sugano, S.S., Shimada, T. & Hara-Nishimura, I., 2013. Enhancement of leaf photosynthetic capacity through increased stomatal density in *Arabidopsis*. New Phytol. 198(3), 757–764.

Tichá, I., 1982. Photosynthetic characteristics during ontogenesis of leaves: 7. Stomata density and sizes. Photosynthetica 16(2), 375—471.

Tramontini, S., Düring, J., Vitali, M., Ferrandino, A., Stoll, M. & Lovisolo, C., 2014. Soil water-holding capacity mediates hydraulic and hormonal signals of near-isohydric and near-anisohydric *Vitis* cultivars in potted grapevines. Funct. Plant Biol. 41(11), 1119–1128.

Wang, Y., Chen, X. & Xiang, C-B., 2007. Stomatal density and bio-water saving. J. Integr. Plant Biol. 49(10), 1435–1444.

Xu, Z. & Zhou, G., 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. J. Exp. Bot. 59(12), 3317–3325.

Stellenbosch University https://scholar.sun.ac.za

Chapter 5

General discussion and conclusions

Stellenbosch University https://scholar.sun.ac.za

CHAPTER V: GENERAL DISCUSSION AND CONCLUSIONS

5.1 Introduction

As mentioned before, stomatal development and functioning have been the focus of numerous studies. There are various environmental and endogenous conditions which alter stomatal development and reactions. This study focused on the effect of four factors on stomatal density and stomatal number per leaf. These factors were date (time), cultivar, leaf position (on the shoot) and observation position (on-leaf). In addition to investigating the main effect of each of the factors on the two variables, the various combinations of interaction effects between them were also investigated. Furthermore a non-destructive microscopy method was used in order to determine the validity of this method as a tool for conducting in-field investigations. Some of these factors have in fact been investigated for *Vitis vinifera* (Düring, 1980; Palliotti *et al.*, 2000; Rogiers *et al.*, 2011) and more recently impression methods have been developed allowing for repeated investigations on the same leaf (Doheny-Adams *et al.*, 2012).

5.2 Outcomes of the study

5.2.1 Aim 1: The use of an adapted microscopy method for conducting stomatal investigations in *Vitis vinifera*

5.2.1.1 Determining whether field microscopy is a viable tool for investigating stomatal density non-destructively

Using the digital microscopy method in conducting stomatal investigations non-destructively, was successful. Since the microscope was able to achieve a magnification of 400x, the stomata were large enough to be counted with ease. Unfortunately the resolution of the microscope was not high enough to allow for clear distinction of epidermal cells. Therefore it was not possible to determine stomatal size and index (the number of stomata in an area in relation to the total number of cells in that area). These two measurements would have added further insight into what developmental differences were present in the study. For this purpose, scanning electron microscopy is still a viable, albeit destructive, alternative method.

There were some problems with this method, which had to be overcome in order to be used effectively and efficiently for stomatal investigation. The ambient light in the field (especially the very bright conditions over midday) impaired stomatal imaging with the digital microscope, since the images produced were not clear enough under these conditions. In order to counteract this, investigations were done standing under an umbrella and the investigated leaf was shaded as far as possible by cupping one's free hand around it and the microscope. The umbrella also helped reduce the glare on the iPad™ screen so that images were seen more clearly. In addition, imaging was not done during the problematic midday period.

The microscope was easy to operate and measurements could be conducted fairly quickly, but assistance was required to capture the images on the iPad™. In-field measurements can be quite strenuous on the body – some leaves occurred in positions which required one to stand in an awkward position during measurements. In general, laboratory microscopy does not render these problems, since one can be seated comfortably and no assistance is required. Many of the microscopy methods used for doing stomatal investigations involve the use of epidermal

Stellenbosch University https://scholar.sun.ac.za

strips or peels. Since the digital microscope can be used on the leaves directly it saves time by eliminating this step.

5.2.1.2 Determining the most effective way of analysing images obtained using field microscopy

The images were analysed using ImageJ (Rasband, 2014). The program has a function for determining areas of shapes and also for counting objects. The method in which standardised (in size and location) grid quadrants were used as the areas in which stomata were counted, was the most unbiased of the three methods tested. It also yielded results that were comparable to that which was reported in literature for *Vitis vinifera*.

5.2.2 Aim 2: Stomatal density and stomatal number per leaf differences in *Vitis vinifera*

The main effects, as well as various combined interactive effects between the different factors, were investigated statistically.

5.2.2.1 Time of season

The main effect of date affected both stomatal density and stomatal number per leaf significantly, but not all dates differed significantly. The same trends were found in interaction effects of date and other factors in various combinations, but the effects seemed to be applicable to only certain cultivars or leaf positions. It was expected that date would have an effect on stomatal density and number, since leaf development occurs over time, but it was expected that there would be more variation between dates.

5.2.2.2 Cultivar

As with date, the main effect of cultivar also caused significant differences in stomatal density and number per leaf between some of the cultivars. With regard to stomatal density, Grenache noir had a significantly higher stomatal value than Pinotage, Shiraz and Cabernet Sauvignon. In the case of stomatal number per leaf, only Shiraz and Cabernet Sauvignon differed significantly.

An interesting observation is that we found similarities in stomatal density between cultivars that tend to respond to drought differently. Shiraz and Grenache noir have been classified as being near-anisohydric and -isohydric respectively (Schultz, 2003; Soar *et al.*, 2006), with Cabernet Sauvignon also being classified as near-isohydric (Tramontini *et al.*, 2014). Near-anisohydric cultivars are not able to maintain a constant leaf water potential during drought stress like near-isohydric cultivars (Schultz, 2003). Cabernet Sauvignon and Grenache noir are thus able to regulate their water status somehow, while Shiraz cannot. This mechanism of control has been found to be predominantly due to regulation in stomatal function [change in aperture] (Schultz, 2003; Soar *et al.*, 2006; Tramontini *et al.*, 2014). This is confirmed by the fact that the near-isohydric cultivars did not necessarily have lower stomatal densities.

5.2.2.3 Leaf position

In general, stomatal density increased as the leaf position moved further away from the shoot (higher density observed for younger leaves). This could be due to the greater sunlight exposure of leaves at the top of the canopy, since light positively affects stomatal density. An increase in stomatal density in turn positively affects photosynthetic rate (Tanaka *et al.*, 2013). Candolfi-Vasconcelos *et al.* (1994) stated that apical leaves in vines had a higher

Stellenbosch University https://scholar.sun.ac.za

photosynthetic rate than the older, basal leaves due to being younger and fully exposed. During the latter part of the season it is the younger leaves that are the most photosynthetically active, allowing for the ripening of the crop and this could be explained by the higher stomatal density observed and the findings by Tanaka *et al.* (2013).

Stomatal number per leaf showed an opposite trend with the number of stomata per leaf decreasing as the leaf position moved further away from the shoot. Apical and Apical 2 leaves had similar stomatal numbers per leaf and this corresponded to their similar leaf size. Thus it can be concluded that leaf size has a greater effect upon determining stomatal number per leaf than did stomatal density. For Cabernet Sauvignon, all leaf positions displayed similar stomatal numbers per leaf and this was once again related to the fact that the leaf sizes of the different leaves for Cabernet Sauvignon did not differ much. This could be related to the low vigour of the Cabernet Sauvignon vines used in this study. In general, the shoots of these vines were shorter than those of the other cultivars (in-field observation) and less vigorous vines tend to have smaller leaves as well. If these vines were more vigorous, with a larger variation between leaf sizes, this trend of little variation between stomatal number per leaf could possibly have been different.

5.2.2.4 On-leaf observation position

In this study stomatal density was found to vary significantly between some of the on-leaf observation positions, but only for the younger, smaller leaves. In previous studies by Düring (1980) and Palliotti *et al.* (2000) the stomatal densities at different positions on leaves were also compared but they found no differences. In the study by Palliotti *et al.* (2000) neither the age of the leaves nor their position on the shoot were specified, thus if we assume that they used more mature leaves lower down on the shoot, this would correspond with the findings of this study. Düring (1980) however, used both young and mature leaves, but the distribution of the three areas investigated differed to that in this study (Figure 56).

Stellenbosch University https://scholar.sun.ac.za

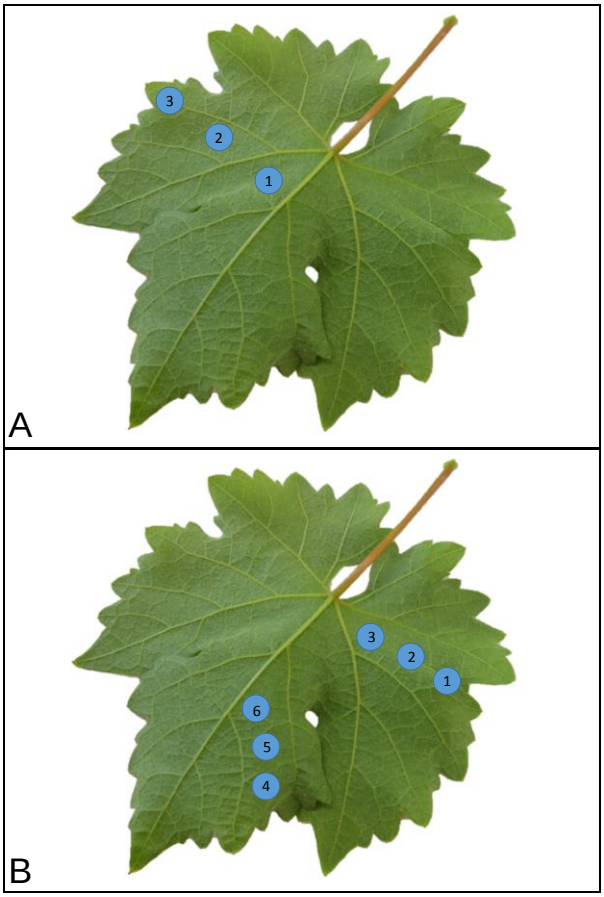

Figure 56 Positioning of the on-leaf observation positions used in A) the study by Düring (1980), and B) this study.

5.2.2.5 Leaf size (for stomatal number per leaf only)

As mentioned previously it was found that leaf size played a very important role in determining stomatal number per leaf. The trends seen between stomatal number per leaf of the different cultivars and leaf positions could always be related to the applicable leaf sizes. This aspect is closely related to the vigour of a vine and it may also be altered by the type of trellis system used. For example, a sprawling canopy tend to produce more shoots, which will lead to the need for a greater distribution of carbohydrates between them. Thus, smaller leaves could be expected in such a canopy in comparison to a hedge trellis (VSP), especially if the latter vines are growing in fertile soil. With increased vigour and leaf size, increased shading may result, but since leaf size seems to affect stomatal number per leaf more than does stomatal density, the shading effect should not lower stomatal number per leaf much.

5.2.2.6 Variation within measurements

There may have been some variation originating from the field repeats (leaves), the image repeats per observation position, as well as the analysis method used (grid quadrants) – this can be regarded as the “residual variance”. The variation in the field repeats and quadrants counted for all the data collected is represented in Figure 57 and Figure 58. Both the field repeats and quadrants showed very little variation within each level. There were no significant differences between the various quadrants, but the stomatal density of field repeat two was higher than for field repeat one.

Stellenbosch University https://scholar.sun.ac.za

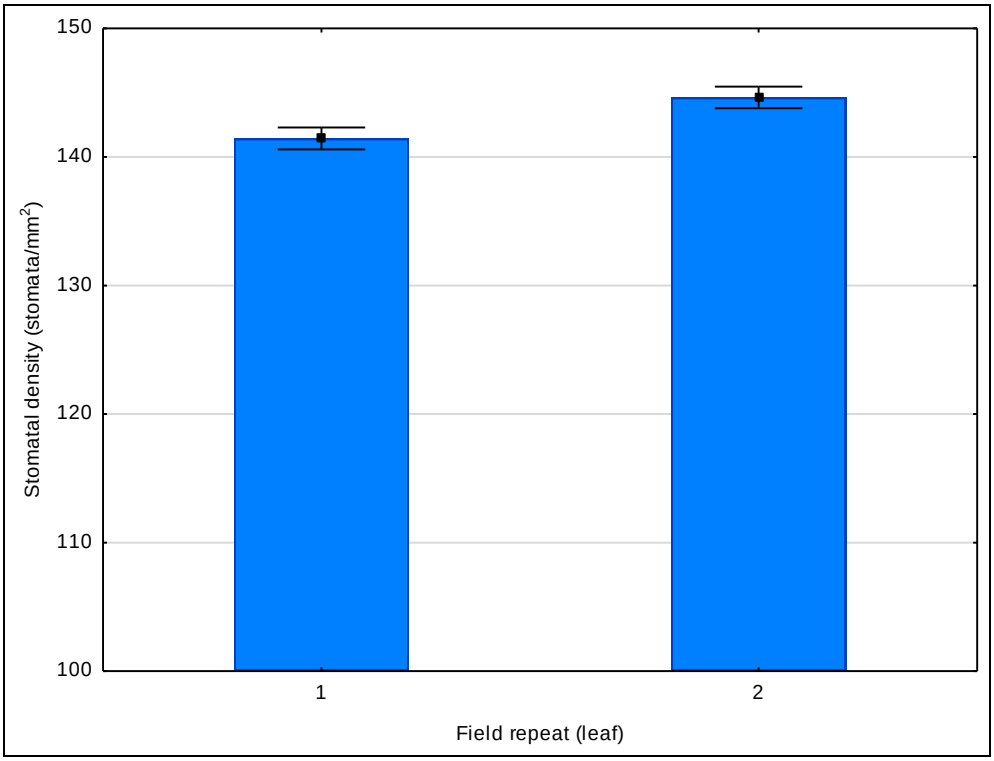

Figure 57 Variation in stomatal density between field repeats (leaves).

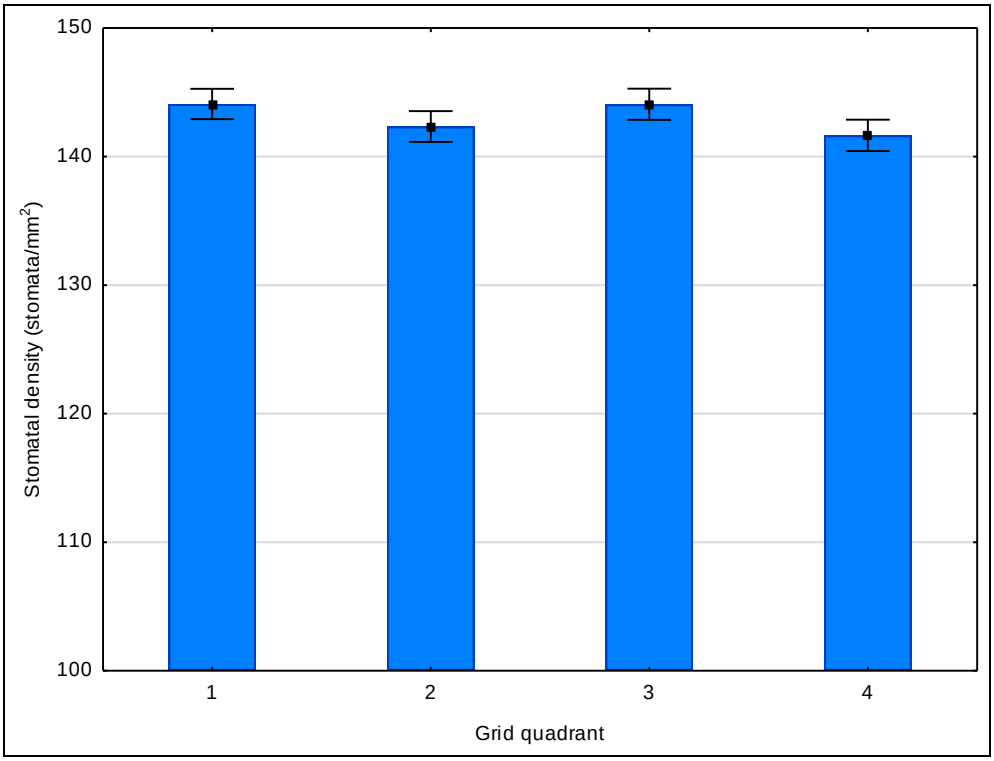

Figure 58 Variation in stomatal density between the counting quadrants; vertcical bars denote 95% confidence intervals.

The variation between image repeats Figure 59 was not large (overlapping confidence intervals), but the within repeat variation was much larger than that of the field repeats and quadrant repeats (larger confidence intervals). On further breakdown of this variance, it was

Stellenbosch University https://scholar.sun.ac.za

noted that this increased within repeat variance may have been caused by the investigations on the youngest leaves (Apical 3) since the confidence intervals were larger for these leaves only (Figure 60).

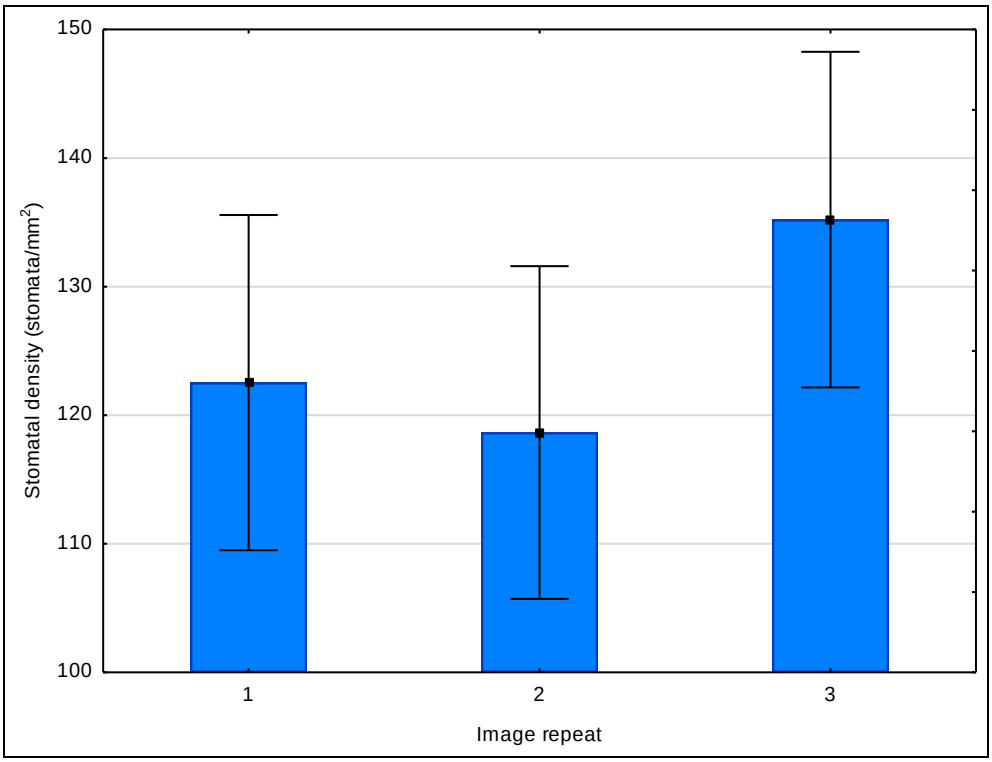

Figure 59 Variation in stomatal density between the image repeats; vertical bars denote 95% confidence intervals.

Stellenbosch University https://scholar.sun.ac.za

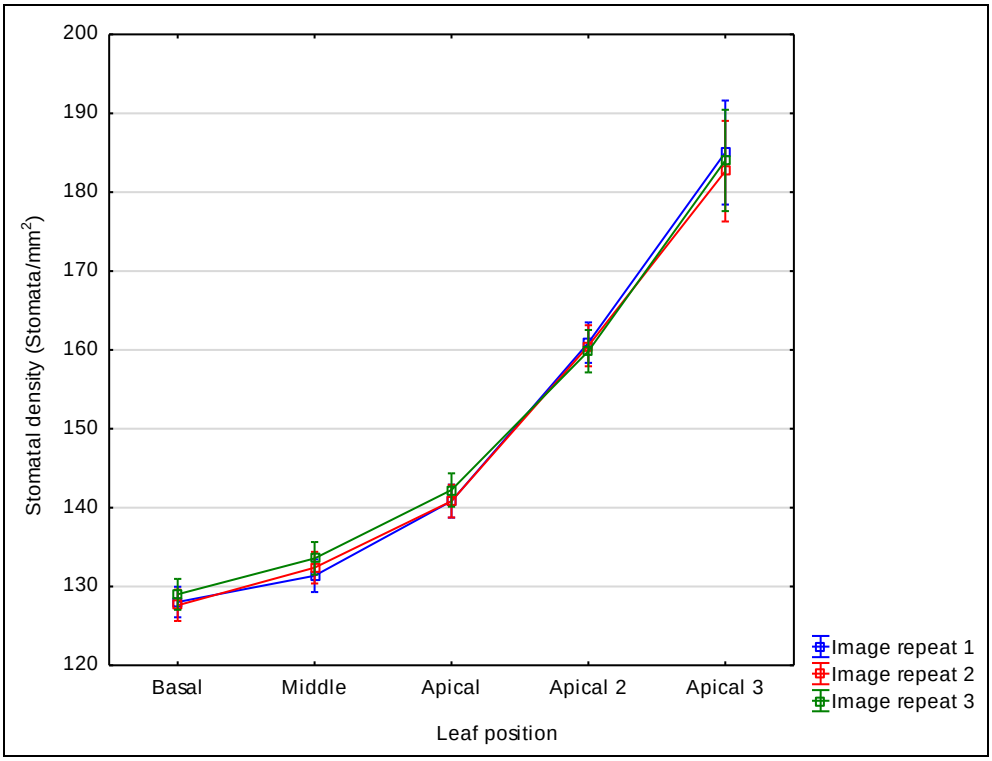

Figure 60 Difference in stomatal density between image repeats of the different leaf positions; vertical bars denote 95% confidence intervals.

The degree of variation between image repeats of stomatal density at the different observation positions for Basal and Apical 3 leaves are shown in Figure 61 and Figure 62 respectively. As noted in a previous section, only Apical 3 leaves showed a variation in stomatal density between observation positions. This can clearly be seen again when comparing the Figure 61 and Figure 62. Please note that the scale in Figure 61 was purposefully chosen to match that of Figure 62 for direct visual comparison between the graphs. Furthermore the variation within each image repeat for the stomatal density at each observation position also differed more, which may have added to the effect of observation position seen in Apical 3 leaves.

Stellenbosch University https://scholar.sun.ac.za

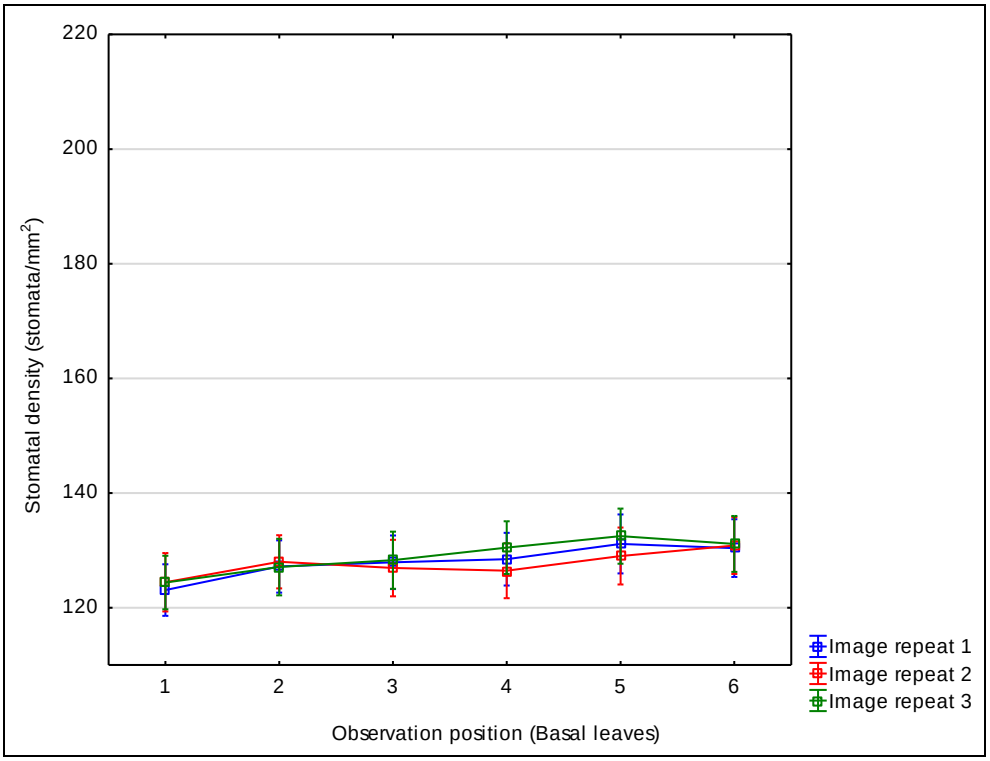

Figure 61 Variation in stomatal density between image repeat at the observation positions (1-6) for Basal leaves; vertical bars denote 95% confidence intervals.

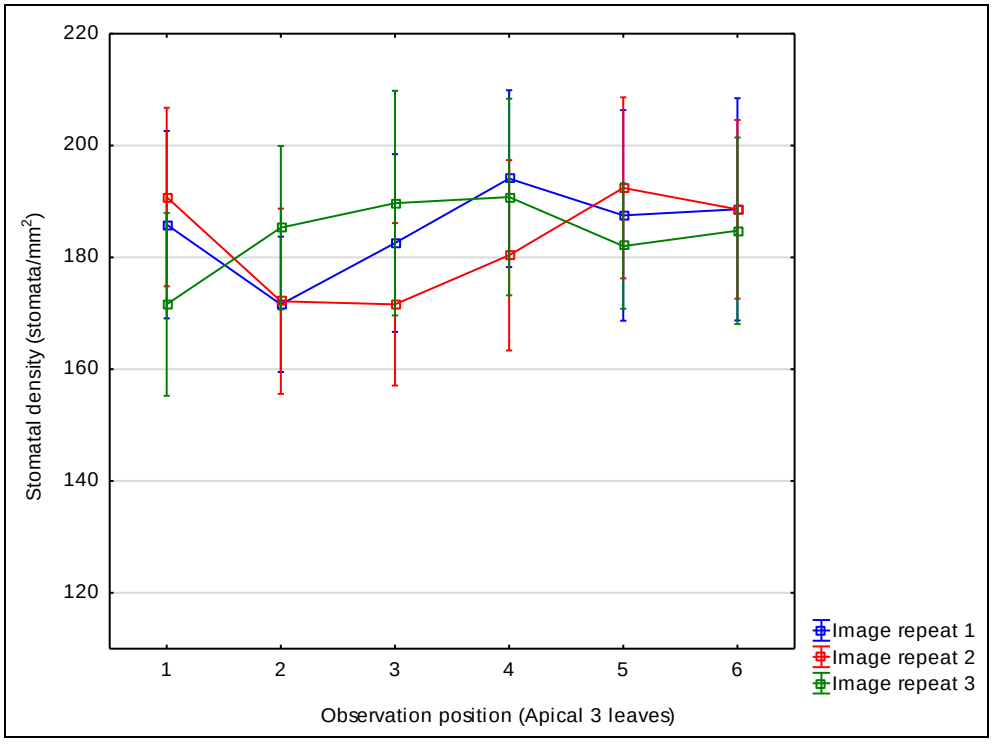

Figure 62 Variation in stomatal density between image repeat at the observation positions (1-6) for Apical 3 leaves; vertical bars denote 95% confidence intervals.

Stellenbosch University https://scholar.sun.ac.za

5.3 Limitations of the study

The study commenced in the latter part of the grapevine's vegetative growth cycle. Thus the limited variation between results found for the dates cannot account for the possible trends that may be present outside of the measured time frame. The smaller the leaves, the more difficult it was to make observations, thus it may not be possible to start observations right after leaf emergence, but possibly closer to the initial growth phase (this may however be possible with different or adapted technology). The damage to younger, translucent leaves that have not yet hardened is also greater and this must be taken into consideration when deciding at what stage to commence future studies. Leaves should also be large enough so that veins do not interfere with observations too much.

It is difficult to determine the exact factor bringing about an observed effect in stomatal density since many of the factors act in combination. There could be other factors, which were not investigated, at play. Thus in-field measurements are very complex and drawing finite conclusions are difficult. The possibility of variance between measurements could further complicate these interpretations (as mentioned in section 5.2.2.6)

The resolution of the digital microscope was not high enough to enable the user to distinguish between individual epidermal cells. As a result stomatal index could not be calculated. Stomatal index, being the number of stomata within an area in relation to the total number of cells (stomatal and epidermal) in that area, often gives a better idea of how stomatal density is affected. It will, for example, indicate whether changes in density are due to a change in cell number or cell size. The lack of definition in the images also prevented the accurate measurement of stomatal size. It would be beneficial to use stomatal size in conjunction with stomatal density or index results, in order to make clearer connections to physiological aspects of leaf and stomatal functioning.

5.4 Perspectives for future research

The study of stomatal density may be more suited to glasshouse studies where certain factors can be controlled. This will make it easier to determine which factors are responsible for bringing about the observed effects. Many of the studies in which the effects of environmental factors on stomatal development and function have been investigated, were conducted in controlled environments, and especially on the model plant *Arabidopsis thaliana*.

It will also be wise to limit the number of factors investigated at a time, since the statistical analysis and interpretation of the results become more complex as more factors become involved. It will also be beneficial if certain aspects, such as light intensity, stomatal conductance and leaf water potential, are monitored so as to be able to verify whether these factors (which cannot be ignored in a field study) may be responsible in bringing about certain responses.

Using a combination of methodologies may also aid in obtaining better, more concise results. Perhaps the newer impression methods (which can be repeated on the same leaf) can be used and the films produced investigated with the digital microscope in the laboratory. Indoor conditions produce better images from the digital microscope, since there is not interfering bright ambient light as in the field. The use of scanning electron microscopy can also be used in conjunction with digital microscopy to cover the aspects of stomatal size and index.

Stellenbosch University https://scholar.sun.ac.za

Technology is continuously improving, thus it may be possible that digital microscopes with a resolution high enough to be able to measure stomatal index and size will soon be available. This will broaden the possibilities of the types of results which can be obtained with this microscopy method. Various types of digital microscopes are also available, some of them serving as an eye-piece which can be used with an ordinary light microscope allowing images to be captured for digital analysis later.

5.5 Literature cited

Candolfi-Vasconcelos, M.C., Koblet, W., Howell, G.S. & Zweifel, W., 1994. Influence of defoliation, rootstock, training system, and leaf position on gas exchange of Pinot noir grapevines. Am. J. Enol. Vitic. 45(2), 173–180.

Doheny-Adams, T., Hunt, L., Franks, P.J., Beerling, D.J. & Gray, J.E., 2012. Genetic manipulation of stomatal density influences stomatal size, plant growth and tolerance to restricted water supply across a growth carbon dioxide gradient. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 367(1588), 547–555.

Düring, H., 1980. Stomatafrequenz bei Blättern von *Vitis*-Arten und -Sorten. Vitis 19, 91–98.

Palliotti, A., Cartechini, A. & Ferranti, F., 2000. Morpho-anatomical and physiological characteristics of primary and lateral shoot leaves of Cabernet Franc and Trebbiano Toscano grapevines under two irradiance regimes. Am. J. Enol. Vitic. 51(2), 122–130.

Rasband, W.S., 2014. ImageJ. U.S. National Institutes for Health, Bethesda, Maryland, USA. Available from: http://imagej.nih.gov/ij/

Rogiers, S.Y., Hardie, W.J. & Smith, J.P., 2011. Stomatal density of grapevine leaves (*Vitis vinifera* L.) responds to soil temperature and atmospheric carbon dioxide. Aust. J. Grape Wine Res. 17(2), 147–152.

Schultz, H.R., 2003. Differences in hydraulic architecture account for near-isohydric and anisohydric behaviour of two field-grown *Vitis vinifera* L. cultivars during drought. Plant Cell Environ. 26(8), 1393–1405.

Soar, C.J., Speirs, J., Maffei, S.M., Penrose, A.B., McCarthy, M.G. & Loveys, B.R., 2006. Grape vine varieties Shiraz and Grenache differ in their stomatal response to VPD: apparent links with ABA physiology and gene expression in leaf tissue. Aust. J. Grape Wine Res. 12(1), 2–12.

Tanaka, Y., Sugano, S.S., Shimada, T. & Hara-Nishimura, I., 2013. Enhancement of leaf photosynthetic capacity through increased stomatal density in *Arabidopsis*. New Phytol. 198(3), 757–764.

Tramontini, S., Düring, J., Vitali, M., Ferrandino, A., Stoll, M. & Lovisolo, C., 2014. Soil water-holding capacity mediates hydraulic and hormonal signals of near-isohydric and near-anisohydric *Vitis* cultivars in potted grapevines. Funct. Plant Biol. 41(11), 1119–1128.